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Compartmental and Regulatory Mechanisms in the Arginine Pathways of *Neurospora crassa* and *Saccharomyces cerevisiae*†

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† This paper is dedicated to the memory of the late William D. Nunn, deceased 1 July 1986.

INTRODUCTION

Arginine metabolism has been studied intensively in many organisms, beginning in earnest with the discovery of the urea cycle in mammals (139). The genetic control of the arginine pathway was the earliest example of the one-gene, one-enzyme relationship in the biochemical genetics of *Neurospora crassa* (206). The elucidation of the pathway in *Escherichia coli* followed soon thereafter, the term "repression" having been coined to describe the regulatory behavior of acetylornithinase (229). The discovery of carbamoyl phosphate in 1955 (131) led to many comparative studies of its metabolism (52, 130, 152). A profound knowledge of arginine metabolism in *N. crassa* and *Saccharomyces cerevisiae* has developed in the last 25 years. The subject justifies a comparative review, because the two organisms solve similar metabolic problems in different ways. It is likely that the fundamental phenomena of compartmentation and regulation in the two organisms can be seen throughout the evolutionary tree, used variously according to the demands of particular lifestyles.

The synthesis of arginine in fungi has three main components: the synthesis of ornithine, the synthesis of carbamoyl phosphate, and the conversion of these two compounds to arginine. The catabolic pathway consists of the hydrolysis of arginine to ornithine and urea, the breakdown of urea to ammonia and carbon dioxide, and the conversion of ornithine to glutamate. A theme to be pursued is how the anabolic and catabolic pathways can each proceed to the exclusion of the other, given ornithine as a common intermediate. A related theme is how carbamoyl phosphate and ornithine are confined to the arginine pathway in the face of enzymes that might divert them to other fates. Both of these matters involve the compartmentation of small molecules by intracellular membranes, which in *S. cerevisiae* is supplemented by elaborate enzyme regulatory mechanisms.

This review describes the enzymology, genetics, and localization of the arginine enzymes of *S. cerevisiae* and *N. crassa*. This is followed by descriptions of vacuolar function, enzyme regulation, and the integration of relevant factors in adaptation to different environments. Metabolic maps (Fig. 1 and 2) and a gene-enzyme directory (Table 1) are given for *N. crassa* and *S. cerevisiae*. Arginine metabolism in these organisms has been reviewed in more limited ways previously (1, 49, 52–54, 56, 57, 66, 128, 185, 248).

BIOSYNTHETIC ENZYMES

Ornithine Synthesis

Overview. The synthesis of ornithine in *N. crassa* and *S. cerevisiae* takes place in mitochondria (10, 45, 127, 238) (Fig. 3 and 4). The path begins with the formation of acetylglutamate from glutamate in the acetylglutamate synthase reaction. Acetylglutamate is converted in several steps to acetylornithine, followed by transfer of the acetyl group to another molecule of glutamate. This regenerates acetylglutamate as ornithine is formed (Fig. 1). Thus most glutamate enters the pathway in the transacetylase reaction, rather than in the more costly acetylglutamate synthase reaction. The latter maintains the level of bound acetyl groups as cells grow and divide, and it counters deacetylation. The synthase reaction is feedback inhibited by arginine, as is the next step in the pathway, acetylglutamate kinase. This assures control of ornithine synthesis if arginine becomes plentiful, regardless of the source of acetylglutamate. The cyclic form of the pathway is found in some bacteria (107), but not in *E. coli* (44a, 232).

In all microorganisms, the acetylated ornithine precursors go through several chemical steps that nonacetylated intermediates go through in proline biosynthesis (Fig. 1). The acetyl group, therefore, chemically isolates these pathways. In fungi the interaction of proline and ornithine metabolism is restricted to the ornithine transaminase reaction, which is a step in arginine catabolism.

***N*-Acetylglutamate synthase.** *N*-Acetylglutamate synthase catalyzes the transfer of the acetyl group of acetyl-coenzyme A (acetyl-CoA) to glutamate to form *N*-acetylglutamate and CoA. The pH optimum of the reaction is about 9.0 for the enzymes of *N. crassa* (114a) and *S. cerevisiae* (258). The apparent K_ms for acetyl-CoA and glutamate are 1.6 and 6.3 mM for the *N. crassa* enzyme. They are also high for the *S. cerevisiae* enzyme (258), although specific values have not been reported. The unusually high pH optimum and high substrate requirements of the enzyme may be related to its location within the small volume of the mitochondria, where it is loosely bound to the inner membrane in both *N. crassa* (114a) and *S. cerevisiae* (127).

N-Acetylglutamate synthase is inhibited by arginine in both *N. crassa* and *S. cerevisiae*. The yeast enzyme is more sensitive to arginine (50% inhibition at 0.02 mM versus 0.16 mM for *N. crassa* in similar reaction mixtures) (258). In addition, the effect of arginine on the *S. cerevisiae* enzyme is intensified by acetylglutamate (which alone has no effect) and CoA (258). In contrast, arginine sensitivity of the *N. crassa* enzyme is not modified by acetylglutamate (114a). These differences have not been correlated with differences in pathway behavior.

N-Acetylglutamate synthase activity is absent in *arg-14* mutants of *N. crassa* (114a) and in *arg2* mutants of *S. cerevisiae* (158). Proof that these loci are structural genes is lacking.

***N*-Acetylglutamate kinase and *N*-acetylglutamyl-P reductase.** *N*-Acetylglutamate kinase and *N*-acetylglutamyl-P reductase convert acetylglutamate to acetylglutamate semialdehyde via an unstable, phosphorylated intermediate (Fig. 1). The activities of both enzymes are among the lowest in the arginine pathway of both organisms, and in *S. cerevi-*

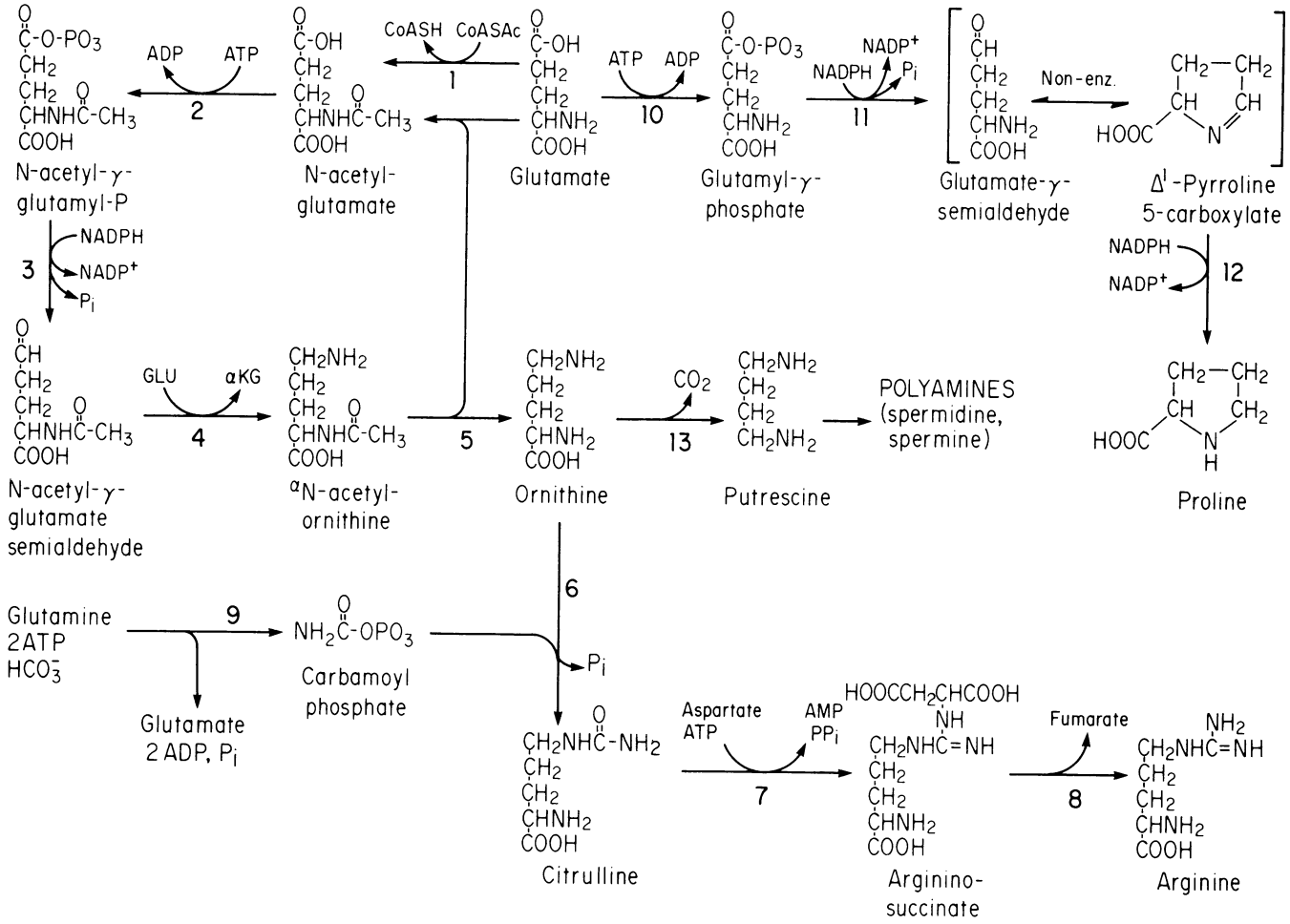

FIG. 1. Anabolic reactions leading from glutamate to arginine, proline, and polyamines. The numbers identify the enzymes listed in Table 1. Nonstandard abbreviations: CoASH and CoASAc, coenzyme A and acetyl-coenzyme A, respectively; GLU, glutamate; α-KG, α-ketoglutarate; Non-enz., nonenzymatic; P_i, inorganic phosphate.

siae the flux in the pathway has been shown to be highly sensitive to variations of the kinase activity (112).

Both enzymes are mitochondrial matrix enzymes in both fungi (127, 234, 259). An early report that the kinase was cytosolic (45) was shown to be erroneous by Wolf and Weiss (259), who showed that a reaction product (pyroglutamate) of a cytosolic activity was confused with the kinase in vitro reaction product (glutamyl hydroxamate) in the earlier work. This problem also prevails in *S. cerevisiae* (25).

The kinase is feedback inhibited by arginine. The *N. crassa* enzyme is half-inhibited by 75 μM L-arginine, but very little by 10 mM ornithine, lysine, citrulline, or carbamoyl phosphate (259). The kinase therefore limits ornithine synthesis in conditions of arginine excess. Feedback-resistant mutants have been isolated (240). The kinase of these mutants has reduced sensitivity to arginine (50% inhibition at 14 mM), and the mutants, unlike the wild type, synthesize ornithine in vivo in the presence of arginine (I. Goodman and R. L. Weiss, submitted for publication). The mutations in these strains are not separable from the kinase structural gene. Given the mitochondrial localization of the kinase, arginine must reach the kinase by transport or facilitated diffusion from the cytosol.

The genetics of the kinase and reductase have been intensively studied in *S. cerevisiae* (125, 170) and *N. crassa* (55, 65, 234). In both organisms, a single complex locus, *arg-6* in *N. crassa* and *ARG5,6* (= *argBC*) in *S. cerevisiae*, encodes the enzyme proteins. The genetic work, together with more recent evidence, suggests that the kinase and reductase are derived from a single polypeptide.

Over 60 mutant alleles of the *S. cerevisiae ARG5,6* locus could be classified into three complementation groups (125, 170). One group (*arg6*, 39 mutants) lacked kinase activity, but had reductase activity. A second group (*arg5*, 18 mutants) had kinase activity and lacked reductase activity. These two groups complemented well. A third group (*arg5,6*, 5 mutants) failed to complement with the first two and lacked both activities. The mutations carried by the first two groups lay in nonoverlapping domains of the genetic locus. There were no nonsense mutants among the kinase$^-$ reductase$^+$ mutants, while six of the kinase$^+$ reductase$^-$ group were nonsense mutants. Significantly, four of the five noncomplementers carried nonsense mutations, and all mapped in the kinase domain. The polarity of the mutants suggested, as a most probable hypothesis, that the locus was expressed as a single messenger ribonucleic acid (mRNA) and a single polypeptide, in which the kinase and reductase are proximal and distal domains, respectively. One of the

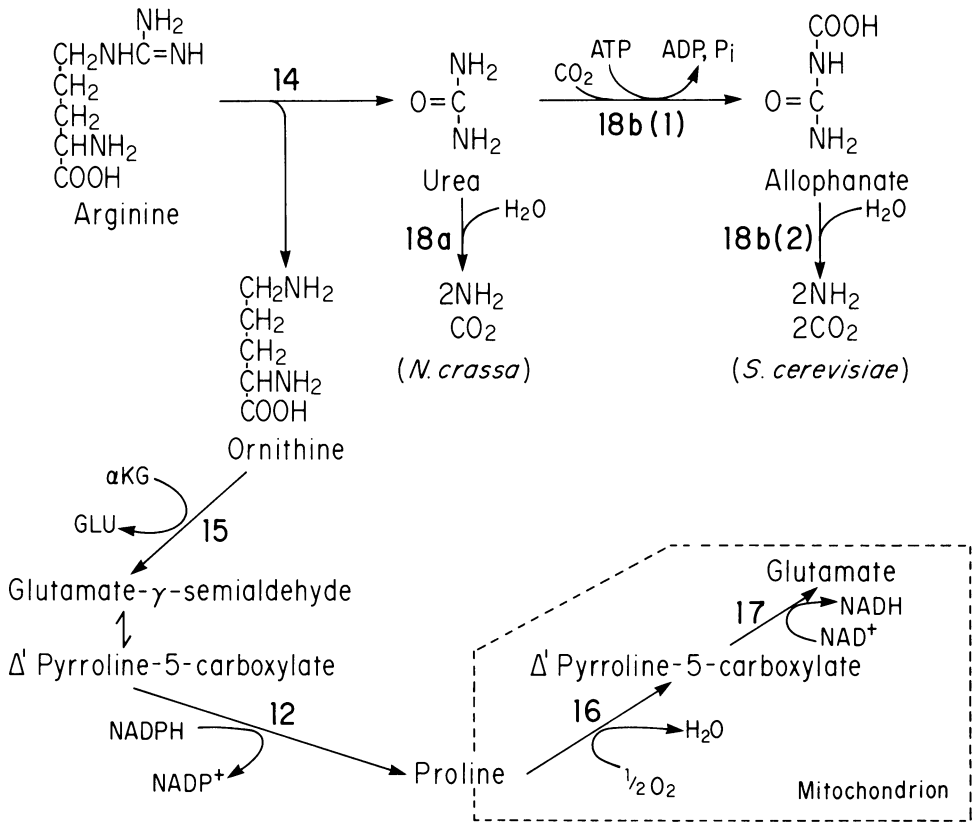

FIG. 2. Catabolism of arginine in *N. crassa* and *S. cerevisiae*. The catabolism of ornithine proceeds via proline in *S. cerevisiae*, and it is probable that this is true in *N. crassa*. Proline catabolism is initiated by proline oxidase (reaction 16), in the inner mitochondrial membrane. Different enzymes (reactions 18a versus 18b) catabolize urea in the two fungi. The numbered enzyme reactions are listed in Table 1. Structural formulas not shown here are shown in Fig. 1; abbreviations are given in the legend to Fig. 1.

few compromising features of the data was that two of the nonsense *arg5,6* mutants, those mapping closest to the reductase ("distal") *ARG5* region, had detectable, albeit weak, reductase function in complementation tests. This conflicts with the rule that there is no reinitiation of translation in eucaryotic mRNAs (138). However, a low level of readthrough of the nonsense codon is not excluded as a mechanism.

In *S. cerevisiae*, the two enzymes specified by the *arg5,6* locus are coordinately controlled over an 85-fold range of activity, in response to the arginine status of the cells and arginine regulatory mutations (170). No other arginine enzyme was regulated coordinately with the kinase or the reductase. In addition, a *cis*-dominant constitutive mutation, *arg5,6-O*^{c}, mapping at the *ARG6* end of the locus was isolated, selecting for overexpression of a leaky *arg5* mutation (125). The mutation affects kinase and reductase equally, but not other arginine enzymes.

Essentially the same picture of the kinase-reductase locus was drawn from a similar study of *N. crassa arg-6* mutations (55, 65, 234). In *N. crassa*, the biochemistry of the enzymes has been more deeply probed.

The enzymes are physically separable in both organisms (170, 234). In *N. crassa*, the kinase is an octamer of 51-kilodalton (kDa) subunits, and the reductase is a dimer of about 40 kDa subunits. The separability of the enzymes was a surprise, because virtually all complex loci of this sort are expressed as mature, multifunctional polypeptides (11, 94). The data required direct tests for a large, polyfunctional precursor bearing both kinase and reductase domains.

A recent study (A. Wandinger, Ph.D. thesis, University of California, Los Angeles, 1985) sought to test the one-mRNA, one-polypeptide hypothesis in *N. crassa*. Both enzymes were purified to homogeneity, and specific antisera were raised to both of them. In vitro translation of mRNA extracted from a derepressed *N. crassa* strain yielded a ca. 90-kDa product which was precipitated with antikinase antiserum. The product, when resolubilized, was reprecipitated with antireductase antiserum. (It was shown that each antiserum failed to recognize the enzyme to which the other antiserum had been raised.) This product was large enough to account for the subunit molecular weights of the kinase and reductase (51,000 and 40,000, respectively). A similar product was shown to accumulate in cells in which mitochondrial protein import had been blocked by respiratory uncouplers. The data as a whole suggest that the precursor is the primary translation product of the *N. crassa arg-6* locus and that it is cleaved to the two enzyme subunits as or after it enters the mitochondria (Fig. 5).

Analysis of the *N. crassa* mutants showed that many kinase$^-$ reductase$^+$ mutants have normal amounts of kinase protein, as expected from the translational polarity model. Some mutants have elevated levels of reductase activity and protein. However, kinase$^+$ reductase$^-$ mutants often had much less than normal kinase activity and protein. This was true for reductase nonsense mutations, which showed the expected deficit in normal-sized reductase protein. The deficiency of kinase protein suggested that the mutant precursor was less stable during its time in the cytosol, or during the mitochondrial entry and processing steps (A. Wandinger,

TABLE 1. Names of arginine-related enzymes and their presumed structural genes in *N. crassa* and *S. cerevisiae*[a]

No. in figures	Systematic name and EC no.	Trivial name used here	Genetic locus (and synonyms)	
			N. crassa	*S. cerevisiae*
1	Acetyl-CoA:L-glutamate *N*-acetyltransferase (EC 2.3.1.1)	Acetylglutamate synthase	*arg-14*	*ARG2* (*argA*)
2	ATP:*N*-acetyl-L-glutamate 5-phosphotransferase (EC 2.7.2.8)	Acetylglutamate kinase	*arg-6*	*ARG5, 6* (*argB, arg6*)
3	*N*-Acetyl-L-glutamyl-5-semialdehyde: $NADP^+$ oxidoreductase (phosphorylating) (EC 1.2.1.38)	Acetylglutamyl-P reductase	*arg-6*	*ARG5, 6* (*argC, arg5*)
4	N^2-Acetyl-L-ornithine: 2-oxoglutarate aminotransferase (EC 2.6.1.11)	Acetylornithine transaminase	*arg-5*	*ARG8* (*argD*)
5	N^2-Acetyl-L-ornithine: L-glutamate *N*-acetyltransferase (EC 2.3.1.35)	Acetylornithine-glutamate acetyltransferase	*arg-4* (*arg-7*)	*ARG7* (*argE*)
6	Carbamoylphosphate: L-ornithine carbamoyltransferase (EC 2.1.3.3)	Ornithine carbamoyltransferase	*arg-12*	*ARG3* (*argF*)
7	L-Citrulline: L-aspartate ligase (AMP forming) (EC 6.3.4.5)	Argininosuccinate synthetase	*arg-1*	*ARG1* (*ARG10, argG*)
8	L-Argininosuccinate::arginine lyase (EC 4.3.2.1)	Argininosuccinate lyase	*arg-10*	*ARG4* (*argH*)
9	Carbon dioxide: L-glutamine amido-ligase (ADP forming, carbamate phosphorylating) (EC 6.4.5.5)	Carbamoyl-P synthetase A (CPS-A)		
		Small subunit	*arg-2*	*CPA1* (*cpaI*)
		Large subunit	*arg-3*	*CPA2* (*cpaII*)
10	ATP:L-glutamate-α-phosphotransferase (EC 1.5.1.12)	Glutamate kinase	*prol-3* (*arg-8*) or *prol-4* (*arg-9*)	*PRO1 or PRO2*
11	L-Glutamate-α-semialdehyde:$NADP^+$ oxidoreductase (phosphorylating) (EC 1.2.1.41)	Glutamyl-P reductase	*prol-4* (*arg-9*) or *prol-3* (*arg-8*)	*PRO2 or PRO1*
12	L-Proline:$NAD(P)^+$ 5-oxidoreductase (EC 1.5.1.2)	Pyrroline-5-carboxylate reductase	*prol-1*	*PRO3*
13	L-Ornithine carboxy-lyase (EC 4.1.1.17)	Ornithine decarboxylase	*spe-1* (*put-1*)	*SPE1*
14	L-Arginine amidinohydrolase (EC 3.5.3.1)	Arginase	*aga*	*CAR1* (*cargA*)
15	L-Ornithine:2-oxo-acid 5-aminotransferase (EC 2.6.1.13)	Ornithine transaminase	*ota*	*CAR2* (*cargB*)
16	L-Amino acid:oxygen oxidoreductase (EC 1.4.3.2)	Proline oxidase	Unknown	*PUT1*
17	1-Pyrroline-5-carboxylate:NAD^+ oxidoreductase (EC 1.5.1.12)	Pyrroline-5-carboxylate dehydrogenase	Unknown	*PUT2*
18a	Urea amidohydrolase (EC 3.5.1.5)	Urease	*ure-1*, *ure-2* *ure-3*, *ure-4*	NA[b]
18b	Urea:carbon dioxide-ligase (ADP forming) (decarboxylating, deaminating) (EC 6.3.4.6)	Urea amidohydrolase		
		Urea carboxylase (1)	NA	*DUR1,2* (*dur1*)
		Allophanate hydrolase (2)	NA	*DUR1,2* (*dur2*)

[a] Complex loci (*ARG5,6*; *DUR1,2*) are resolved to component functions in the older gene designations of *S. cerevisiae*. All genes are nuclear.
[b] NA, Not applicable.

Ph.D. thesis). In *S. cerevisiae*, by contrast, nonsense reductase mutants had normal kinase activity (170).

If the two enzymes are aggregated in the mitochondrion, it might allow channelling and protection of the labile intermediate, acetylglutamyl-γ-phosphate, in the course of ornithine synthesis. Assuming a 1:1 stoichiometric ratio of subunits, the mature enzymes would fully aggregate if four reductase dimers complexed with each kinase octamer. This possibility cannot yet be evaluated. The issue is important, however, because it highlights the question of whether mitochondrial matrix enzymes, such as those of the Krebs cycle, are specifically organized, or whether their catalytic efficiency derives solely from their high local concentration (207).

N^2-Acetylornithine transaminase, N^2-acetylornithine:glutamate acetyltransferase, and acetylornithinase. N^2-Acetylornithine transaminase and N^2-acetylornithine:glutamate acetyltransferase are required for the formation of ornithine from acetylglutamate semialdehyde. The specificity and significance of acetylornithinase is not known.

The transaminase is a mitochondrial matrix enzyme (45, 127). It requires pyridoxal phosphate to convert acetylglutamate semialdehyde to N^2-acetylornithine, using glutamate as an amino donor. Transaminase mutants of *N. crassa* and *S. cerevisiae* (see Table 2) are tight auxotrophs, indicating the essential role of the enzyme in the pathway. The acetyl function on the amino group of *N*-acetylglutamate semialdehyde prevents the cyclization of the molecule seen in proline synthesis (Fig. 1). The acetylated intermediates are thereby committed to ornithine synthesis.

In the acetyltransferase reaction, the acetyl group of acetylornithine is transferred to glutamate, and ornithine is liberated. Acetylglutamate is therefore regenerated upon the formation of ornithine. As noted earlier, the reaction is the major entry point of glutamate in arginine synthesis, and mutants of this enzyme are almost complete auxotrophs (55). It is possible that the slight leakiness of these mutants reflects the small amount of acetylglutamate that can be made in the acetylglutamate synthase reaction, combined

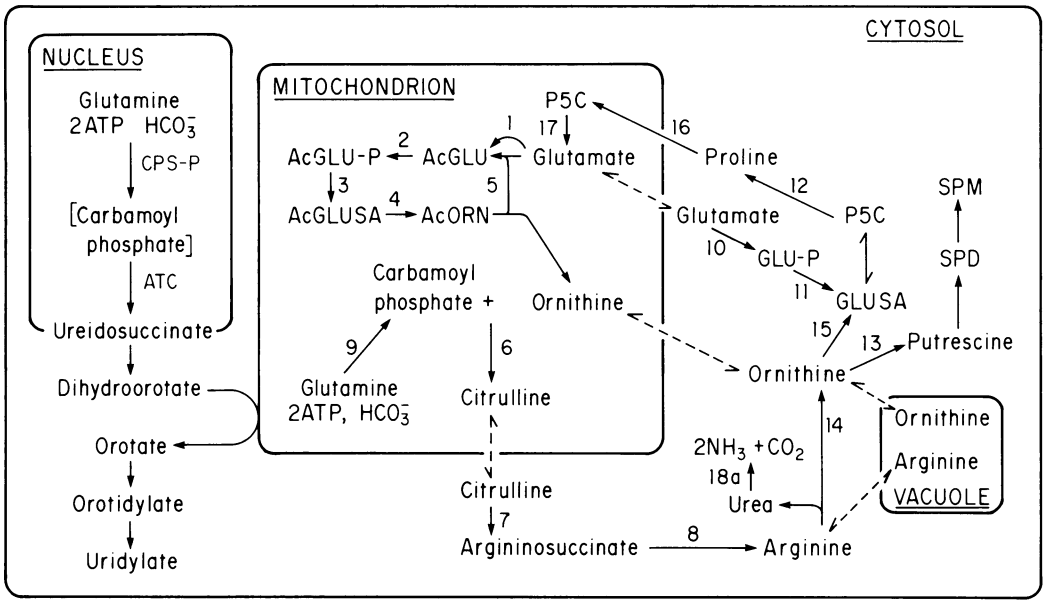

FIG. 3. Organization of arginine, pyrimidine, proline, and polyamine metabolism in *N. crassa*. The locations of reactions 16 and 17 have not been proved for *N. crassa*, but are shown as in *S. cerevisiae*. The numbered reactions are found in Fig. 1 and 2 and are listed in Table 1. Abbreviations: ATC, aspartate carbamoyltransferase; CPS-P, pyrimidine-specific carbamoyl-P synthetase; Ac-, acetyl; GLU, glutamate; GLUSA, glutamate semialdehyde; ORN, ornithine; P5C, pyrroline-5-carboxlyate; SPD, spermidine; SPM, spermine.

with weak nonenzymatic or enzymatic deacetylation of the derived acetylornithine (69, 119; R. H. Vogel and H. J. Vogel, Genetics **48**:914, 1963). The transacetylase is located in the mitochondrial matrix (45, 127).

Early work with the formation of ornithine in *N. crassa* and *S. cerevisiae* began with the assumption that acetylornithine was hydrolyzed to ornithine and acetate as in *E. coli*. The initial experiments actually revealed an acetylornithinase in both fungi (70; R. H. Vogel and H. J. Vogel, Genetics **52**:482, 1965). However, the yeast enzyme had very low affinity for acetylornithine, and in view of the essentiality of the transacetylase shown by mutants of both fungi (70; Vogel and Vogel, Genetics **52**:482, 1965), no further study of acetylornithinase has been reported. Its location in the cell is not known.

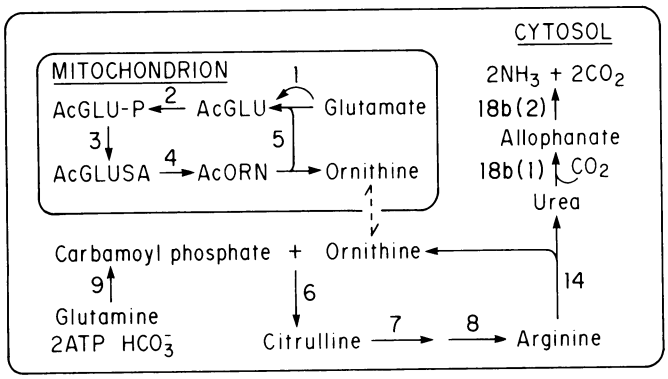

FIG. 4. Organization of selected arginine enzymes in *S. cerevisiae*, showing nonmitochondrial locations of ornithine carbamoyltransferase (reaction 6) and CPS-A (reacton 9), and the existence of the urea amidohydrolase reactions in this organism (reactions 18b). Other reactions are organized as in Fig. 3.

Carbamoyl Phosphate Synthesis

Enzymology. Two carbamoyl phosphate synthetases (CPSs) are found in animals and fungi, one specific for the arginine pathway (CPS-A) and one specific for the pyrimidine pathway (CPS-P). The two enzymes are regulated independently and are specified by different genes (49, 52, 67, 130, 141, 152). In fungi, CPS-A has a small and a large subunit, encoded by unlinked genes.

CPS-A of *N. crassa* and *S. cerevisiae* uses the amide nitrogen of glutamine, 2 mol of adenosine triphosphate (ATP)-Mg, and bicarbonate to form carbamoyl phosphate, inorganic phosphate, adenosine diphosphate (ADP), and glutamate (63, 141, 147, 193). Both CPS-A's will use ammonia as an N donor in place of glutamine in vitro, though with lower efficiency. The enzyme reaction requires free Mg^{2+} and K^+. The kinetic characteristics of the enzymes from the two organisms are given in Table 2.

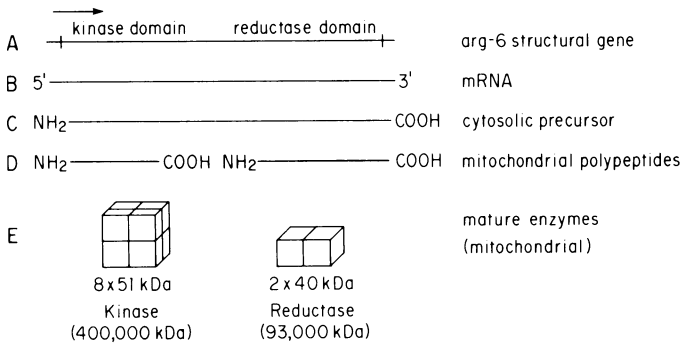

FIG. 5. Steps in the expression of the *arg-6* gene of *N. crassa*, leading to active acetylglutamyl kinase ("kinase") and acetylglutamyl-P reductase ("reductase"). The arrow in A indicates the direction of transcription.

TABLE 2. Kinetic characteristics of CPS-A from *N. crassa* (48, 63) and *S. cerevisiae* (141, 193, 194)

Characteristic	*N. crassa*	*S. cerevisiae*
K^+ optimum (mM)	20	100
Free Mg^{2+} requirement	Yes	Yes
K_m (glutamine) (mM)	1.6	1.25
K_m (ammonium) at pH optimum (mM)[a]	16	75
K_m (ATP-Mg) (mM)[b]	~1–2	1–2
K_m (HCO_3) (mM)	4.0	4.3
pH optimum	7.9	7.6
V_{max} (glutamine)/V_{max} (NH_3)	~1.3	~10
Inhibition by arginine or precursors	No	No

[a] Assuming ammonia to be the true substrate of the "ammonium-dependent" reaction, the K_m values for the base would be 0.88 and 1.3 mM for the *N. crassa* and *S. cerevisiae* enzymes, respectively.

[b] Given for the glutamine-dependent reaction; the dependence is not strictly Michaelian.

The roles of the two subunits of CPS-A are similar to those of many glutamine amidotransferases, including the CPSs of bacteria (191). The small subunit splits glutamine in the course of the reaction, procuring the amide nitrogen for use in a later step. The large subunit carries out all other catalytic functions. These involve the formation of a carbonate-phosphate anhydride, its conversion to an enzyme-bound carbamate, and the phosphorylation of the latter to form carbamoyl phosphate (257). Ammonium, at high concentration, can replace glutamine as a substrate, and the large subunit alone catalyzes the ammonia-dependent reaction. The high K_m for ammonium (63, 193) reflects in part the fact that the true substrate of this reaction is ammonia (28). The V_{max} of the ammonia-dependent reaction is slightly lower than that of the glutamine-dependent reaction in *N. crassa* and much lower for the *S. cerevisiae* enzyme (Table 2). These data indicate that, in the glutamine-dependent reaction, the small subunit channels the amide nitrogen (as unprotonated ammonia) to the large subunit more efficiently than free ammonium/ammonia can enter the catalytic cycle from the surrounding medium.

Owing to extreme instability, neither the *N. crassa* nor the *S. cerevisiae* holoenzyme has been purified. Only recently has the purification of the more stable large CPS-A subunit of *N. crassa* been accomplished (174, 234a). Although subunit dissociation occurs in the course of inactivation of both fungal enzymes, dissociation may follow, rather than cause inactivation of glutamine-dependent activity by heat (63, 193).

Structure and genetics of CPS-A. The small subunit of CPS-A is encoded in the *CPA1* gene of *S. cerevisiae* and the *arg-2* gene of *N. crassa*; the large subunit is encoded in the *CPA2* and *arg-3* genes of the respective organisms (Table 1) (49, 62, 141, 193). In both organisms, the two CPS-A genes are unlinked. Molecular weight estimates of the *N. crassa* enzyme, based on gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, are 176,000 for the holoenzyme, 125,000 to 130,000 for the large subunit, and 45,000 for the small subunit, (62, 63, 174). In *S. cerevisiae*, the respective values are 175,000 (by gel filtration [193]), 124,000, and 45,000, the last two deduced from the nucleotide sequence of the cloned genes (150, 241).

In both organisms, extracts of mutants lacking one subunit complement in vitro those lacking the other with respect to the glutamine-dependent reaction (192, 193; R. H. Davis and J. L. Ristow, unpublished experiments). It has been impossible to purify the small-subunit complementing activity of *N. crassa*. The *S. cerevisiae* small subunit survives gel filtration and diethylaminoethyl-cellulose chromatography, but it too is quite unstable (193). In the case of *N. crassa*, the glutamine-dependent activity restored through complementation is even less stable than the native holoenzyme, while in *S. cerevisiae* the two are similar. The data suggest that the subunits have higher affinity for one another in *S. cerevisiae* than in *N. crassa*. This is correlated with the different localizations of CPS-A in the two organisms: the *N. crassa* enzyme is mitochondrial, while that of *S. cerevisiae* is cytosolic (Fig. 3 and 4). The requirement for subunit affinity would be much less in the small volume of the mitochondrion than in the larger volume of the cytosol.

Antisera to the *N. crassa* CPS-A large subunit cross-react with corresponding subunits of CPS of *E. coli* and of CPS-A of yeasts (174). The same antisera recognize the CPS I of the rat, and the CPS-Ps of *N. crassa* and *S. cerevisiae*, which are found in the nucleoli in a multienzyme complex with aspartate carbamoyltransferase (10, 149, 172, 255).

Localization and regulation. CPS-A of *N. crassa* is located in the mitochondrial matrix (62, 63, 238). The large or small subunit could be found within the mitochondria of mutants lacking the other subunit, and the subunit present was normally controlled (62). The data imply that the polypeptides enter mitochondria separately and aggregate only after insertion. Recently, a cytosolic 135-kDa precursor of the large subunit has been visualized by immunoblot techniques. After it is pulse-labeled in vivo with [^{35}S]methionine in conditions blocking the entry of the precursor into mitochondria, it can be chased into its mature form in the mitochondria, a process requiring mitochondrial energy. It is of interest that this process is notably slower in a strain carrying a C-terminal nonsense mutation lacking approximately 30 kDa of the normal 130-kDa protein. This implies an involvement of the C-terminal end of this large protein in the localization or maturation process (S. A. Ness, Ph.D. thesis, University of California, Los Angeles, 1985).

The ratio of CPS-A subunits is of interest in connection with maturation and regulation of the enzyme. In *N. crassa*, the large subunit is not repressible, and its activity rises three- to fivefold upon arginine starvation (45, 62). By contrast, the glutamine-dependent activity is repressed 4- to 10-fold by arginine and rises 10-fold upon arginine starvation over values characteristic of cells grown in minimal medium (45). This is correlated, qualitatively, with the intensity of staining of the small subunit in two-dimensional gels (62). The small subunit is therefore probably limiting at least at the lower end of the range of regulation, behaving in effect as a rate-determining cofactor of the large subunit. It is not excluded that the aggregation of the small and large subunits in the mitochondria is regulated in some manner by arginine.

In *S. cerevisiae*, CPS-A and ornithine carbamoyltransferase are cytosolic (225), in contrast to most other eucaryotes. The metabolic consequences of this will be discussed in a later section. Regulatory studies showed that extracts of arginine-grown cells have little glutamine-dependent activity, and only a small amount of ammonium-dependent activity, compared with extracts of cells grown in minimal medium. It might be inferred that both activities are repressed by arginine. However, if the assay of the large subunit is performed by adding unlimiting amounts of a *cpa2* extract (containing small subunit) and assaying the glutamine-dependent reaction that appears, much more large subunit is apparent (192). This suggests that the large subunit becomes active in the presence of arginine only when it aggregates with the small subunit. With this taken into

account, the small and large subunits are regulated similarly to those of *N. crassa*. In addition, the large subunit may positively regulate the small subunit, because less small subunit is found in *cpa2* mutants than in the wild type. However, this may be due simply to protection of the small subunit from proteolysis, as suggested by Piérard et al. (192).

The short-term control of CPS-A activity is not understood. In *N. crassa*, no intermediate of the pathway, nor arginine itself, inhibits CPS-A (63). Arginine does not inhibit CPS-A of *S. cerevisiae* (248). Even combinations of possible effectors have no effect in *N. crassa*. The initial interpretation of these findings was that the high degree of repression of the small subunit took the place of feedback inhibition (45). However, later studies in *N. crassa* yield evidence that arginine, directly or indirectly, rapidly influences the accumulation (and thus possibly the synthesis) of carbamoyl phosphate (Davis and Ristow, unpublished experiments). This will be discussed in a later section (see "Biochemical Integration of Arginine Metabolism").

Channelling. Channelling of carbamoyl phosphate takes place in *N. crassa*. The carbamoyl phosphate made in the mitochondrion is normally confined to use in arginine synthesis by the mitochondrial membrane. Similarly, carbamoyl phosphate made by the multifunctional complex of CPS-P and aspartate carbamoyltransferase in the nucleolus is confined as an enzyme-bound intermediate to pyrimidine synthesis (255, 256). Only when one of the carbamoyltransferases is absent or defective can carbamoyl phosphate be diverted to the other pathway. In *S. cerevisiae*, the cytosolic location of CPS-A allows the product of the enzyme to be shared with the pyrimidine pathway. The behavior of the *N. crassa* system is one of the clearest examples known of metabolic channelling by organellar or "molecular" (148) compartments. Because it has been extensively reviewed (49, 52, 53), it will not be discussed further here.

Conversion of Ornithine to Arginine

Conversion of ornithine to arginine requires three enzymes, ornithine carbamoyltransferase, argininosuccinate synthetase, and argininosuccinate lyase. In *N. crassa*, ornithine carbamoyltransferase is in the mitochondria (238), while in *S. cerevisiae* it, like CPS-A, is cytosolic (225). In both organisms, the last two enzymes of the pathway are cytosolic (127, 238).

Ornithine carbamoyltransferase. Ornithine carbamoyltransferase catalyzes the transfer of the carbamoyl group from carbamoyl phosphate to the δ-amino group of ornithine, forming citrulline and inorganic phosphate. The reaction is strongly in favor of citrulline, but it is reversible even in vivo under certain conditions (158).

Ornithine carbamoyltransferases of *N. crassa* and *S. cerevisiae* are trimers of ~37-kDa subunits and have estimated native molecular weights of ~110,000 (5, 190). The *S. cerevisiae ARG3* gene has been sequenced, and the deduced molecular weight of the product is 37,842 (R. Huygen and M. Crabeel, personal communication). The enzymes differ in ways that may be related to their different localizations. In keeping with the more alkaline environment of the mitochondrion versus the cytosol, the pH optimum of the pure *N. crassa* enzyme is pH 9.5 (5), while that of the pure *S. cerevisiae* enzyme is pH 8.5 (83). The pH optima are 0.5 U less for both enzymes in a partially purified state (e.g., 47, 127). The K_ms for ornithine and carbamoyl phosphate are higher at the optimal pH values than at lower pH values, and these values too vary greatly (0.1 to 5.0 mM for ornithine; 0.02 to 2.5 mM for carbamoyl phosphate) with the state of purity of the enzymes (5, 47, 83). However, the ornithine carbamoyltransferase of *S. cerevisiae* displays ornithine substrate inhibition above 5 mM (83, 164). This is a correlate of a novel control mechanism, to be discussed in a later section (see "Biochemical Integration of Arginine Metabolism"), by which ornithine carbamoyltransferase is inhibited by aggregation with arginase during the transition from anabolic to catabolic conditions (165).

A mutational variant of *N. crassa*, *arg-12*^{s}, has only 3 to 5% of normal activity (46, 47). The deficiency does not impose a growth requirement for arginine, although the enzymes of the arginine pathway are derepressed. The enzyme has a 10-fold-higher K_m for ornithine and an abnormally low K_m for carbamoyl phosphate (0.011 mM) at pH 8.8 (L. G. Williams, Genetics **77**:s70, 1974). The greater affinity for carbamoyl phosphate may compensate partially for the low V_{max} of the enzyme, particularly if the mitochondrial pH is 8.0 or higher.

The characteristics of the ornithine carbamoyltransferase of this mutant were used to identify *arg-12* as the structural gene for the enzyme (64). The corresponding locus for ornithine carbamoyltransferase of *S. cerevisiae* is *ARG3* (41). Both loci have been cloned (41, 93). The yeast gene was expressed in *E. coli* only after deletion of a sequence 5′ to the ornithine carbamoyltransferase coding region had created an effective promoter function for it in *E. coli* (41). The same deletion removed sequences required for expression in yeast cells. Finally, linked, *cis*-dominant mutations impairing the arginine-specific control behavior of ornithine carbamoyltransferase have been isolated in *S. cerevisiae* (158) (see "Regulation of Anabolic Enzymes").

Argininosuccinate synthetase and argininosuccinate lyase. In the reaction catalyzed by argininosuccinate synthetase, a quaternary complex of citrulline, ATP-Mg, aspartate, and enzyme is formed, followed by the release of adenosine monophosphate (AMP), PP_i-Mg^{2+}, and argininosuccinate (196). The probable hydrolysis in vivo of pyrophosphate (which severely inhibits the bovine enzyme) renders the reaction physiologically irreversible. The *N. crassa* and *S. cerevisiae* enzymes have similar pH optima around pH 7.8 (114, 233). The enzyme from *S. cerevisiae* has been studied in detail (114) and resembles the mammalian enzyme (114, 196) in many respects. Arginine is inhibitory to the enzyme of both species (114, 233). This may or may not have regulatory significance.

The structural genes for the enzyme are *arg-1* in *N. crassa* (175) and *ARG1* in *S. cerevisiae*. Substantial intragenic complementation takes place among mutations of the gene in both organisms (113, 114, 220), a finding that led *S. cerevisiae* workers initially to think that *ARG1* was a complex locus, *ARG1,10*. Study of the physical properties of the enzyme (114) reveals that it is a tetramer of identical 49-kDa subunits and, thus, the product of a single gene. (The gene symbol *ARG10* has thus been vacated.)

The argininosuccinate formed by the synthetase is hydrolyzed to arginine and fumarate in a reversible reaction catalyzed by argininosuccinate lyase. Pure argininosuccinate lyase of *N. crassa* has a K_m of 0.2 mM for argininosuccinate in the forward reaction and K_ms of 0.5 and 0.8 mM for fumarate and arginine, respectively, in the reverse reaction (27). The native molecular weight is 176,000 and is constituted of multiple, but an unknown number of, subunits. Mutations of the gene for the enzyme, *arg-10*, display intragenic complementation, consistent with a multiple subunit structure (25, 88, 175). In *S. cerevisiae*, the locus

governing the lyase activity is *ARG4*. *ARG4* deoxyribonucleic acid (DNA) has been cloned and shows an open reading frame capable of coding for a 52-kDa subunit, consistent with the 53-kDa value based on other experiments (6). The codon usage in this gene indicates that it has moderate expression in *S. cerevisiae*.

ARGININE CATABOLISM

Overview

Arginine degradation to glutamate (Fig. 2) ultimately yields 2 mol of ammonia in the arginase and urease reactions, 1 mol of amino nitrogen (as glutamate) in the ornithine transaminase reaction, and the main carbon skeleton as another molecule of glutamate. Arginine is a relatively good nitrogen source for *S. cerevisiae* and *N. crassa*. Arginine and ornithine transaminase are both cytosolic. After uptake into the cell, arginine is hydrolyzed to ornithine and urea. Urea is converted to carbon dioxide and ammonia by different mechanisms in the two fungi. In *N. crassa*, urease hydrolyzes urea to CO_2 and $2NH_3$, while in *S. cerevisiae* an enzyme complex, urea amidolyase, yields the same net products after an ATP-requiring carboxylation of urea.

Ornithine is transformed in the transaminase reaction to glutamate-γ-semialdehyde by loss of the α-nitrogen to α-ketoglutarate. The semialdehyde is transformed to proline, which, in excess, is degraded to glutamate via the mitochondrial proline oxidase and pyrroline-5-carboxylate dehydrogenase reactions. This unusual coupling of proline biosynthesis and arginine catabolism, proved so far only for *S. cerevisiae*, may be widespread in fungi.

Uptake of Arginine

N. crassa and *S. cerevisiae* have similar permeases for the basic amino acids. Two permeases, defined by mutation, kinetics, and specificity, mediate uptake of arginine in *N. crassa*. These are the basic amino acid permease (183, 197, 221) and the general amino acid permease (68, 179, 182, 195), deficient in strains carrying the *bat* and *pmg* mutations, respectively. The basic amino acid permease has high affinity for arginine ($K_m = 2.4 \times 10^{-6}$ M) and lesser affinities for ornithine, lysine, and histidine (183). This permease is found in young, rapidly growing cells. In contrast, the general system mediates uptake of all amino acids, both D and L isomers (182). Its activity is highest in older, C-starved (182) and N-starved (86) cells, and it is nitrogen repressed. It has very high affinity for arginine ($K_m = 2 \times 10^{-7}$ M) and high activity. The maintenance of the general amino acid permease depends upon a normal intracellular amino acid pool (221). The general system functions as a major element of nitrogen assimilation (see "Regulation of Catabolic Enzymes").

Much is known about the kinetics of permeases of *S. cerevisiae*, owing largely to the work of M. Grenson (99). Here again, there is a basic amino acid permease, specified by the *CAN1* gene, which recognizes all basic amino acids (K_m [arg] = 10^{-5} M) (105). In contrast to *N. crassa*, there is also a distinct, very specific lysine transport system specified by another gene, *LYP1* (98). A general amino acid permease, specified by the *GAP1* gene, is also present. Like that of *N. crassa*, it transports many amino acids, both D and L, has high affinity for arginine (K_m [arg] = 7.6×10^{-6} M), and is nitrogen regulated (104). Several mutations that pleiotropically affect this and other permeases are known (103, 216).

Transport in fungi is not well understood mechanistically. Two features of the process are noteworthy. First, an amino acid within the cell causes, at some level, "transinhibition" of further uptake of that or other amino acids. The specificity of transinhibition is similar to that of the permease functions. (The term transinhibition was used by Pall and Kelly [184] and by Grenson et al. [102] to indicate inhibition of a transport system by molecules accumulated by that transport system. Cooper [31] defines it as inhibition of one species' transport by intracellular molecules of a different species entirely. The usage of Pall and Grenson is retained here.) Second, the uptake of amino acids, particularly basic amino acids, is accompanied by uptake into the vacuole. The relative rates of uptake into the cell and into the vacuole lead to a high concentration of arginine in the cytosol as long as arginine is available in the medium (236). This allows the arginase reaction to proceed while entry takes place, but when the medium is depleted of arginine the vacuolar fraction remains protected as a nitrogen reserve (239). The vacuolar sequestration of arginine may be one reason why its efflux from the cell, even by exchange with arginine in the medium, is slow.

Arginase and Ornithine Transaminase

Arginase. In *S. cerevisiae*, arginase, like ornithine carbamoyltransferase, is a trimer, having an M_r of ~114,000 with subunits estimated to be ~39,000 (190). (The deduced amino acid sequence, however, yields a subunit M_r of 35,616 [213].) The enzyme has a high K_m for arginine (2 to 5 mM), appropriate for a catabolic enzyme (168). The enzyme has a metal requirement, usually satisfied by Mn^{2+} in assays in vitro. However, kinetic comparisons and pH profiles of native arginase and variously substituted metalloarginases led Middelhoven and co-workers to conclude that Fe^{2+} was the cofactor of the *S. cerevisiae* and rat liver enzymes (168, 169). The Fe form of the enzyme is much less active than the Mn form. If Fe^{2+} is the usual form of the enzyme, it would rationalize in part the observation that arginase activity is far greater in the usual in vitro assay with Mn^{2+} than it appears to be in living cells. An impression of high activity is further accentuated by measuring the reaction at pH 9.5, the optimum, rather than at the cytosolic pH of about 7. No confirmation of the Fe^{2+} metalloenzyme has been presented.

N. crassa arginase is a hexamer of 240 kDa, with subunits of approximately 38 kDa (K. Borkovich, Ph.D. thesis, University of California, Los Angeles, 1985). Its K_m for arginine is estimated at 5 to 25 mM (R. H. Davis, unpublished experiments; Borkovich, Ph.D. thesis). Immunoprecipitated enzyme has little or no associated Fe^{2+} but contains Mn^{2+}. The Mn^{2+}/arginase ratio of such precipitates is about 0.2:1.0, and Mn^{2+} is only loosely bound to the enzyme, unlike true metalloenzymes (the dissociation constant, drawn from a possible model of the reaction mechanism, is in the micromolar range) (Borkovich, Ph.D. thesis).

Arginase is present in minimal medium-grown *N. crassa* cells. No arginine is catabolized during growth of cells in minimal medium (51), despite a finite cytosolic arginine pool on the order of 0.1 mM (135). This can be attributed to a combination of factors: (i) the sigmoid concentration-velocity curve of native arginase (66), (ii) the suboptimal pH of the cytosol for enzyme activity, (iii) a limitation of Mn^{2+} (Borkovich, Ph.D. thesis), and (iv) the low arginine concentration.

Arginase-deficient mutants of *S. cerevisiae*, *car1*, were selected for their inability to grow on arginine as a nitrogen

source. Mutants of *N. crassa*, *aga*, were selected from *pro-3* mutants as variants unable to use arginine to satisfy their proline requirement (59, 171). *N. crassa aga* mutants are prototrophic, but they acquire a polyamine requirement on arginine-supplemented medium (59). The polyamine pathway begins with ornithine, which is decarboxylated to form putrescine (Fig. 1 and 3). In arginaseless cells grown in arginine, de novo ornithine synthesis is feedback inhibited, and the *aga* mutant cannot form ornithine by catabolism. A polyamine dependence therefore develops. In *S. cerevisiae* arginine does not inhibit *car1* mutants. This is probably because the requirement for polyamines in *S. cerevisiae* is so low that special media are required to demonstrate it, even in ornithine decarboxylase-deficient mutants (29, 247).

Ornithine-δ-transaminase. Ornithine-δ-transaminase was first studied intensively in *N. crassa* in relation to the biosynthesis of arginine and proline (230). It was thought that it might be a biosynthetic enzyme, converting glutamate-γ-semialdehyde to ornithine, but mutant studies did not support this idea (87). The acetylated pathway of ornithine synthesis was soon discovered, depriving ornithine transaminase of a place in arginine biosynthesis (Vogel and Vogel, Genetics **48**:914, 1963). When mutants lacking the enzyme were found to be prototrophic, it was certain that the enzyme was in fact a catabolic enzyme (60). In *N. crassa*, the enzyme has a pH optimum of 7.4 and K_ms for ornithine and α-ketoglutarate of about 2 mM (231). The cyclization of the product to Δ^1-pyrroline-5-carboxylate makes the reaction physiologically almost irreversible (Fig. 2) (230).

Recently, certain higher plant ornithine transaminases were found to use the alpha, not the delta, amino group of ornithine (166). The product of the reaction cyclized to Δ^1-pyrroline-2-carboxylate, indistinguishable by previous tests from the 5-carboxylate. A recent study in *S. cerevisiae* showed that the product of ornithine transaminase was in fact Δ^1-pyrroline-5-carboxylate, and thus the delta amino group of ornithine was donated to α-ketoglutarate (19).

S. cerevisiae mutants lacking ornithine transaminase, *car2*, were selected as unable to use ornithine as a sole nitrogen source. The corresponding *N. crassa* mutants, *ota*, were selected by a scheme based on the compartmental behavior of ornithine (50, 60). The *arg-12*^{s} mutant of *N. crassa* has a severe bottleneck in citrulline synthesis at the ornithine carbamoyltransferase reaction, owing to low activity and low affinity for ornithine. Because *arg-12*^{s} imposes no growth requirement, it clearly does not prevent use of endogenous ornithine (made in mitochondria) for citrulline synthesis. However, the mutation blocks use of *exogenous* ornithine as a citrulline and arginine precursor. Thus the double mutant *arg-5*, *arg-12*^{s} will not grow on ornithine, as does the single mutant carrying only the *arg-5* mutation. If the double mutant is used to select variants able to grow on ornithine (as an arginine source), the majority are deficient in ornithine transaminase activity. The ability of the derived triple mutants to grow on ornithine stems from the much higher pool of ornithine that can develop in such cells, high enough to enter mitochondria and satisfy the high substrate requirement of the mutant ornithine carbamoyltransferase (50).

Urea Degradation

In *N. crassa*, the urease reaction yields CO_2 and two molecules of NH_4^+ by hydrolysis of urea (206). The enzyme, as far as is known, is similar to the classic ureases of plants (e.g., jackbean urease). Mutations imparting a urease deficiency are known at four loci, two (*ure-1*, *ure-2*) close to one another, but not adjacent, on linkage group V (137) and two (*ure-3*, *ure-4*) distantly linked on linkage group I (109). In all four sets of allelic mutants, one or more with a heat-sensitive enzyme was found, suggesting that conceivably all genes contribute to the structure of the enzyme (9, 109). The tests were done with crude extracts; no work on the pure protein has been reported, nor have tests for remediation by Ni^{2+} been done. Ni^{2+} is a covalently bound metal in classic ureases, and high levels of Ni^{2+} were found to reverse some urease deficiencies in *Aspergillus nidulans* (151). This may reflect involvement of other genetic loci in the maturation of the enzyme. *N. crassa* urease is a large enzyme, like that of jackbean urease (M_r = 489,000), and the involvement of four loci is not as unlikely as it might at first seem.

In *S. cerevisiae*, urea is broken down by a two-step sequence called urea amidohydrolase (198–201, 242–244) (Fig. 2). In the first reaction, urea is carboxylated to allophanate (200) in a HCO_3^--, ATP-Mg-, and biotin-dependent reaction catalyzed by urea carboxylase (200, 242). In the second reaction, catalyzed by allophanate hydrolase, allophanate is hydrolyzed to two molecules each of CO_2 and NH_4^+. The initial reaction has high affinity for urea (K_m of ca. 0.4 mM) and CO_2 (K_m of 1 mM) (244), and CO_2 is effectively catalytic in the overall reaction (242). The investment of an ATP in the reaction and the nitrogen repression of the enzyme (14, 76) imply that the overall reaction has a nitrogen-scavenging role.

The complex genetic locus encoding urea amidohydrolase has components denoted *DUR1* and *DUR2* (32). The two components were tightly linked, and certain mutants with mutations in the *DUR2* domain (for allophanate hydrolase) lacked both of the urea amidohydrolase activities. This implied the existence of a multifunctional polypeptide, the allophanate hydrolase domain being translation proximal. Indeed, both enzyme activities were associated with a monomeric, 204-kDa polypeptide defined by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (211). In this work, rapid immunoprecipitation from crude extracts was done to prevent proteolytic cleavage of the large polypeptide.

Urea amidohydrolase is induced by allophanate (245) (see "Regulation of Catabolic Enzymes"). In the process, the achievement of a constant differential rate of increase of allophanate hydrolase activity preceded that of urea carboxylase by 2 to 6 min (209). The delay was shown to be the time required for the addition of biotin to the urea carboxylase domain, a process mediated by biotin holoenzyme synthetase (200, 209).

Fate of Δ^1-Pyrroline-5-Carboxylate

S. cerevisiae strains lacking proline oxidase do not degrade ornithine to glutamate, despite their having ornithine transaminase and pyrroline-5-carboxylate dehydrogenase (19). Further study revealed that proline was an obligatory intermediate in ornithine and arginine breakdown. In the actual pathway, Δ^1-pyrroline-5-carboxylate is first reduced to proline by the cytosolic proline-biosynthetic enzyme pyrroline-5-carboxylate reductase. Proline oxidase, a mitochondrial enzyme, yields an intramitochondrial Δ^1-pyrroline-5-carboxylate pool which is further oxidized to glutamate (20). This circuitous route of Δ^1-pyrroline-5-carboxylate degradation reflects the mitochondrial location of pyrroline-5-carboxylate dehydrogenase (20). The only

way its substrate can enter mitochondria is via proline and the proline oxidase reaction (Fig. 2 and 3). In *N. crassa*, the small amounts of ornithine catabolized by cells grown in minimal medium goes to proline, not glutamate (135). At high levels of ornithine, glutamate also appears (135). Thus, as far as they go, the data for *N. crassa* are consistent with the scheme described for *S. cerevisiae*.

THE VACUOLE

Isolation and General Characteristics

Investigators of fungal arginine metabolism were initially troubled by the large intracellular pools of basic amino acids. If the cellular arginine and ornithine were dissolved homogeneously in cell water, their concentrations would each exceed 8 mM. This would be expected to overwhelm feedback mechanisms and would lead to catabolic enzyme induction. Early tracer work, however, suggested that amino acids in general were compartmented into separate pools: one large, with low metabolic turnover, and one small, with high turnover (37, 38). In 1973 Wiemken and Nurse (253), working with *Candida utilis*, and Weiss (235) and Subramanian et al. (208), working with *N. crassa*, showed directly that the large pools of arginine and certain other amino acids were largely confined to the vacuole, sequestered from cytosolic amino acid pools used for protein synthesis.

In *S. cerevisiae*, current methods of vacuole isolation (251) involve digesting the cell wall by incubation with snail gut enzymes, followed by osmotic, mechanical, or polybase lysis of the cell membrane. In all such cases, the osmoticum is adjusted to preserve the integrity of the vacuole. The vacuoles are purified by sedimentation or flotation in sucrose or Ficoll gradients. The *S. cerevisiae* vacuole is large and fragile, and it is not easy to apply the isolation techniques to cells at all stages of culture. In addition, incubation of cells in snail gut enzyme for over an hour at 28°C (251) may be accompanied by metabolic changes affecting vacuolar contents. *N. crassa* vacuoles are small and less fragile than vacuoles of *S. cerevisiae* (42, 227). They can be isolated after rapid mycelial breakage with a glass bead homogenizer (43). The method minimizes opportunities for metabolic changes during cell disruption, and the method can be applied to any stage of growth.

Vacuoles of both fungi contain a variety of hydrolases, such as proteases, glycosidases, nucleases, and phosphatases (155, 157, 227, 254). Vacuoles therefore have the functions of animal lysosomes, a matter reviewed elsewhere (156). Considerable protein degradation during certain phases of the yeast life cycle proceeds in the vacuole, and arginine may be derived by this route at some stages (210).

Most or all of the inorganic polyphosphate of *S. cerevisiae* and *N. crassa* is found in vacuoles (44, 122, 224), although disagreement prevails about how much might be associated with the cell wall (140, 222). Vacuolar polyphosphates have from 3 to 50 P residues in both fungi (82, 176, 227). A large portion of the basic amino acids (over 95%) are found in vacuoles of both fungi. These neutralize, in part, the strong negative charge of polyphosphate. However, a considerable amount of spermidine is found in *N. crassa* vacuoles, where it is tightly complexed with polyphosphate (42, 186). No comparable studies of spermidine have been reported in *S. cerevisiae*. In both fungi, some divalent, inorganic cations, notably Mg^{2+}, are found. Their amounts depend (at least in yeasts) upon the ions available to the cell (42, 181). Very little of the predominant cellular monovalent cation (K^+) was found in the vacuole of *N. crassa*.

The vacuolar pH of *N. crassa* and *S. cerevisiae* cells grown in minimal medium is about 6.1 ± 0.4 (143, 176). The pH of the cytosol, by contrast, is neutral (143, 177). The pH gradient is maintained by a distinct, vacuolar proton-pumping adenosine triphosphatase (ATPase) found in both organisms (17, 18, 180). As noted below, the proton motive force is the energy source for basic amino acid transport.

Amino Acid Pools

Two main methods are used for determining the size of "cytosolic" and vacuolar pools. One is by differential lysis, a technique devised first by Schlenk et al. (205). Intact cells are exposed to a basic protein or polybase, which renders the cell membrane permeable and liberates nonvacuolar, low-molecular-weight compounds. Vacuolar contents are then liberated by strong osmotic shock after the cells have been washed free of cytosolic contents. While this method overestimates the ratio of cytosolic to vacuolar amino acids, it has been valuable in the study of intracellular amino acid distribution (160, 253, 261).

The other method, used in *N. crassa* (135, 208), was based upon earlier radioactive tracer methods (37, 38, 108, 154, 230, 262) and is more accurate in the determination of cytosolic pool size. In its first use (208), a small amount of arginine, of high specific radioactivity, was added to cells, and the dilution of radioactive arginine by endogenous arginine as it is incorporated into protein was determined. The dilution factor was very small, indicating that the cytosolic arginine pool was very small (208). The rest was in the vacuole, an inference confirmed by direct isolation (235). The isotopic method has been applied to arginine, proline, ornithine, and other amino acids. Its rationale and use are described more fully in the last section of this review.

The large pools of basic amino acids of *N. crassa* and *S. cerevisiae* were strongly associated (>90% in most cases) with the vacuole under all conditions (160, 227, 235, 253). About 70% of the cationic charge equivalents in the vacuoles of *N. crassa* grown in minimal medium are accounted for by basic amino acids, mainly arginine and ornithine (42). The persistence of basic amino acids during the isolation and purification of vacuoles may reflect in part the impermeability of the membrane to this class of molecule (42, 44) and in part the charge interaction of arginine with polyphosphate (42, 82, 224) (see below).

Substantial amounts of all classes of amino acids were associated with vacuoles of *S. cerevisiae*, and the association depended upon the strain, the medium, and the nutritional status of the organism (160). Systematic studies have not been done in *N. crassa* for all amino acids. The fraction of the neutral and acidic amino acids that were associated with the organellar pellet in cell fractionation studies of *N. crassa* suggested some sequestration of these amino acids in the vacuole (227). Further purification of vacuoles led to diminishing amounts of these amino acids, however. A more critical study of glutamine-, alanine-, citrulline-, and proline-N by nuclear magnetic resonance spectroscopy showed that these molecules were predominantly cytosolic (134, 142, 143). For the last two amino acids, this was in accord with the tracer study of Karlin et al. (135). As far as they go, the nuclear magnetic resonance data are in sharp contrast to *S. cerevisiae*, where glutamine, at least, was predominantly vacuolar in cells grown in a similar medium (252). Because the data for amino acid localization were collected in quite different ways for the two organisms, and because the environment strongly influences the location, the apparent

differences between the two fungi should be regarded with caution.

Freshly isolated vacuoles of *S. cerevisiae* rapidly lost arginine until a 1:1 stoichiometric ratio of polyphosphate-P/arginine was established (82). It was suggested that, in vivo, the transport of arginine into vacuoles might simply be a matter of charge distribution, in which polyphosphate, an impermeant macromolecule, combined with selective permeability of the vacuolar membrane, trapped basic amino acids in the vacuole (156). Equilibrium dialysis with vacuolar extracts of *S. cerevisiae* (82) and synthetic polyphosphate (44, 82) showed that this was not unrealistic. The appeal of the hypothesis was strengthened by the observation that the ratio of vacuolar polyphosphate-P/basic amino acids was 1:1 in both organisms in many conditions (44).

By manipulation of arginine and phosphate metabolism, disparate ratios of basic amino acids and polyphosphate could be achieved (44, 82). This showed that neither component was required in stoichiometric amounts for vacuolar accumulation of the other. The conclusion was extended to vacuoles of *N. crassa* isolated from phosphate-starved or arginine-grown cells. These vacuoles, which were large and fragile and had little polyphosphate, contained a large amount of arginine and other basic amino acids (42). Arginine did not escape from them, and no alternate, ion-trapping polyanion which might take the place of polyphosphate was found. Therefore, arginine was retained by the *N. crassa* vacuole (in contrast to the isolated *S. cerevisiae* vacuole) not by interaction with a polyanion, but by the impermeability of the vacuolar membrane.

The importance of polyphosphate to arginine retention was diminished further in *N. crassa* by the finding, noted above, that almost half the polyphosphate of the vacuole was complexed with spermidine (42, 186). Polyphosphate does reduce the osmotic pressure of basic amino acids, because considerable vacuolar swelling takes place upon growth in arginine as a sole nitrogen source, or after depletion of polyphosphate (42). Thus, while polyphosphate is not obligatory for accumulation of amino acids, it may strongly influence the amount of basic amino acids that can be accumulated in the vacuole of both fungi.

Arginine Transport by Vacuoles

Early studies of arginine transport by isolated vacuoles of *S. cerevisiae* by Boller et al. revealed an exchange process in which uptake of radioactive arginine was accompanied by efflux of endogenous arginine (12). The process was mediated by an arginine-binding element with an apparent K_m of 30 μM, in contrast to the transport function of the intact spheroplast (K_m [arg] = 1.5 μM). The arginine taken up was retained if external arginine was removed. Ornithine and lysine were not inhibitory to uptake, but D-arginine, L-histidine, and D-histidine were. Low concentrations of proteases could destroy transport activity after an initial activation (81).

Active transport was demonstrated in vacuolar membrane vesicles of *S. cerevisiae* by Ohsumi and Anraku (180). Transport was accumulative and driven by an ATP-generated proton gradient. The ATPase involved was distinct from mitochondrial and plasma membrane ATPases by the criterion of inhibitor sensitivity. The arginine transport function had an apparent K_m (arg) of 0.6 mM, some 20-fold higher than the exchange function described by Boller et al. (12). The specificity of transport was high. Separate systems for H^+/substrate antiport for arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine, and isoleucine-leucine were discerned (202). Further studies (203) demonstrated arginine-histidine exchange in which the apparent K_m for arginine entry was 0.1 mM. It is not known which of these systems, if any, is responsible for the arginine exchange discovered by Boller et al.

The K_m for transport of amino acids by vacuolar membrane vesicles of *S. cerevisiae* is higher by two or three orders of magnitude than the corresponding plasma membrane systems. This undoubtedly reflects the intracellular environment in which the vacuole functions, with cytosolic amino acid pools on the order of 0.05 to 1 mM.

The enzymology of the vacuolar membrane ATPase of *N. crassa* has also been investigated (17, 18). This ATPase is associated with membranes of pure vacuoles, and, as in *S. cerevisiae*, it has different inhibitor sensitivity than the ATPases of the mitochondrion and plasma membrane. The coupling of the vacuolar membrane proton gradient to arginine transport was demonstrated (C. R. Zerez, R. L. Weiss, C. Franklin, and B. J. Bowman, J. Biol. Chem., in press). The apparent K_m for arginine was 0.4 mM, again similar to the *S. cerevisiae* system. The transport of arginine was not inhibited by ornithine or lysine, but D-arginine was slightly inhibitory at high concentration. Thus, and again like yeasts, the amino acid specificity and the affinity of this system are both different from basic amino acid transport systems of the plasma membrane.

The behavior of vacuolar arginine pools in living *N. crassa* mycelia is consistent with the energetics of vacuolar accumulation so far determined in vivo. Cells grown in minimal medium and labeled with [^{14}C]arginine were treated with inhibitors of glycolysis, inhibitors or uncouplers of respiration, or both (74, 75). Transport of cytosolic arginine into the vacuoles was blocked by inhibitors of respiration or uncouplers, which considerably lowered the energy charge. However, vacuolar arginine was not lost under these conditions, indicating that metabolic energy was not needed for retention of arginine. This is consistent with the observation (42) that vacuoles retained arginine well in vitro (see above). Other manipulations showed that energy status is involved in mobilization of arginine from vacuoles (75). For instance, a glutamine deficiency is the metabolic signal denoting nitrogen starvation. Upon glutamine starvation, arginine is mobilized from vacuoles of *N. crassa* and is catabolized. Respiratory inhibitors and uncouplers block mobilization of arginine. A second finding was that inhibitors of glycolysis, which lowered the ATP pool but did not lower the energy charge, led to loss of arginine from the vacuole in vivo, even though glutamine was not seriously depleted. The loss was again blocked by respiratory inhibitors (75). The glycolytic derivative acting as a signal for retention or release of vacuolar arginine has not been identified.

REGULATION OF ANABOLIC ENZYMES

Overview

N. crassa displays subdued regulation of arginine enzyme activity. Most biosynthetic enzymes become elevated about fourfold upon arginine starvation (45). The catabolic enzymes have a high basal level and are induced three- to fourfold upon arginine addition (60). Of the biosynthetic enzymes, only CPS-A activity is repressed below the levels seen in cultures grown in minimal medium (45). For these reasons, compartmentation, feedback inhibition, and enzyme design have unusually prominent roles in *N. crassa*.

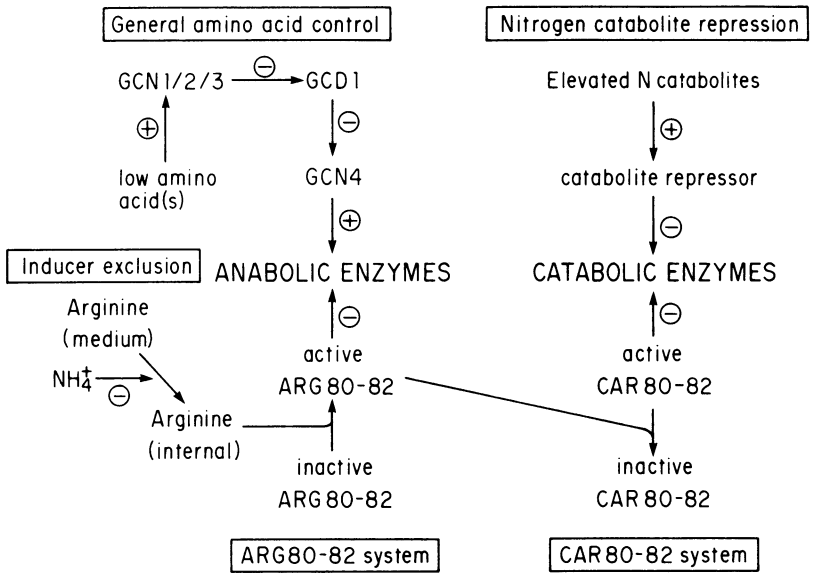

FIG. 6. Models of regulatory gene action in arginine metabolism in *S. cerevisiae*. The circled plus and minus signs indicate the action of each wild-type gene product or metabolite(s) upon the immediately following element in the sequence. These models are useful for understanding the phenomenology, but are not definitive descriptions of the systems. For instance, it is not certain that the nitrogen catabolite repression system acts by a negative control at the structural gene level, nor is it clear at what level the *ARG80-82* system interacts with the *CAR80-82* system and the catabolic enzymes. See text for further discussion. In *N. crassa*, the nitrogen catabolite control system appears to involve a positively acting gene product (*nit-2*), and no systems corresponding to *ARG80-82* and *CAR80-82* have been found.

S. cerevisiae, in contrast, displays large amplitudes of regulation of most anabolic and catabolic enzymes. Five regulatory systems have been defined here, two being wholly absent in *N. crassa*. Biosynthesis is controlled by general amino acid control, by another, arginine-specific, system devoted to six of the nine enzymes of the pathway, and by a third system devoted solely to the small subunit of CPS-A (the first and second are depicted in Fig. 6). Catabolism is controlled by a specific arginine catabolic regulatory system, by nitrogen catabolite repression, and by ammonia-induced inactivation of the permeases (Fig. 6). These systems are all superimposed upon the compartmental and feedback mechanisms we see in *N. crassa*. A list of *trans*-acting genes involved in regulation, together with earlier, synonymous symbols, is given in Table 3.

General Amino Acid Control

Behavior of the system and its mutants. Starvation of *N. crassa* or *S. cerevisiae* for an individual amino acid, or subjecting cells to concentrated supplements of certain single amino acids, leads to derepression of enzymes of many amino acid biosynthetic enzymes (179; I. B. Barthelmess, Mol. Gen. Genet., in press). The response helps to redress imbalances of amino acids in the cytosol and provides a long-term solution to problems the vacuolar storage pool can correct only transiently. Feedback inhibition fine-tunes the derepression response. General amino acid control, discovered in *N. crassa* (22–24), has been analyzed in great detail in *S. cerevisiae*. The reader is referred to later papers cited (e.g., reference 116) and reviews (97, 111) as an entree into the area. ("Cross-pathway control of amino acid synthesis" was used to describe this phenomenon when it was originally discovered in *N. crassa*, and workers in this organism still use this term. In this review, I use "general amino acid control," as it is used by *S. cerevisiae* workers.)

General amino acid control involves derepression of many of the enzymes of the arginine, lysine, tryptophan, histidine, isoleucine, valine, and leucine biosynthetic pathways upon starvation of cells for any of these amino acids (2, 22–24, 71, 90, 136, 179, 260). The involvement of other amino acids and the response of other pathways have not been excluded. Only the end product of the deficient pathway will restore normal enzyme levels. The system is not directly influenced by the choice of nitrogen source. Derepression can be evoked by pathway inhibitors such as 3-amino-1,2,4-triazole (a histidine analog). In the case of the arginine pathway of *S. cerevisiae*, all 10 polypeptides are synthesized at a higher rate (159), and this is probably true of *N. crassa* (45).

Genetic and molecular analyses of the *S. cerevisiae* system reveal a complex cascade regulatory circuit (116, 117) (Fig. 6). The regulatory protein that interacts with enzyme structural genes is the *GCN4* gene product (121). Mutations of this gene (*gcn4*) are phenotypically null (nonderepressible), implying a positive action of the *GCN4* product on the structural genes. At least one copy of the hexanucleotide TGACTC (or a closely related sequence) in the 5′ nontranscribed regions of the affected genes is required for general amino acid control (73, 118). The mRNA of *GCN4* has a long leader which in wild-type cells leads to poor translation of the coding sequence (115, 217). The negative effect of the leader is exerted only when a second gene, *GCD1*, is intact. When the *GCN4* leader is missing, or when the *GCD1* gene is mutant, translation of *GCN4* mRNA proceeds, and derepression of the structural genes results. Therefore, *gcd1* mutations are constitutive. Finally, the action of *GCD1* is antagonized by three further genes, *GCN1*, *GCN2*, and *GCN3* (117, 188), if one or more amino acids are limiting. If one of these genes is mutant, or if amino acids are plentiful and well balanced, repression of the biosynthetic enzymes takes place. To summarize this cascade in another way: amino acid limitation activates *GCN1*, *GCN2*, and *GCN3* to block the action of *GCD1*; this permits *GCN4* mRNA to be translated, and the *GCN4* protein stimulates transcription of amino acid biosynthetic genes.

Mutants carrying *gcd1* are unable to initiate DNA synthesis at 35°C. They therefore have the phenotype of an early, temperature-sensitive, cell cycle lethal mutant (260). This suggests that the general amino acid control system might address the cell division control system, possibly by arresting growth at G1 upon a serious amino acid imbalance, a situation in which the *GCD1* function is blocked.

Other mutations of this system which are poorly understood are *Gen*^{c}, which causes induced, rather than repressed, levels of biosynthetic enzymes in rich medium (159). The *tra5* mutations are very similar in regulatory phenotype but are not allelic to *gcd1* mutations. A *gcn5* (= *aas104*) mutation complementing with *gcn1-gcn4* is also known but has not been placed in the regulatory scheme (188). Some mutations may have their effect by triggering the general control system indirectly.

In *N. crassa*, mutations of only one general amino acid control gene, *cpc-1*, are known so far (2, 55). The mutants are nonderepressible for diagnostic enzymes, similar to the *gcn* series of *S. cerevisiae*. They were found as mutations which rendered the bradytrophic *arg-12*^{s} mutant completely dependent upon added arginine. A range of functional impairment is suggested by variation in severity of effect of different *cpc-1* alleles. In addition to impairing the derepression normally evoked by amino acid starvation or addition of 3-amino-1,2,4-triazole, *cpc-1* mutations diminish the levels of enzyme seen in cells grown in minimal medium (2; F. Stuart, D. J. Porteous, H. J. Flint, and H. J. Kacser, J. Gen. Microbiol., in press). This leads one to ask whether a complete loss of *cpc-1*$^+$ function would lead to a complete inability to make any enzymes of the affected pathways, a presumed lethal phenotype. One allele of the series, *cpc-1*$^{j-5}$ was isolated in a strain carrying a simultaneously induced, second mutation, *slo* (3). Strains carrying the *cpc-1*$^{j-5}$ allele only were inviable. The role of the *slo* mutation and the basis of its interaction with the *cpc-1* is not known, but a nonsense suppressor action for *slo* has not been excluded. The indispensability inferred for the normal allele of the *cpc-1* mutation may reflect a role in establishing finite basal levels of amino acid biosynthetic enzymes. More such alleles of the gene are needed to explore this possibility further, but to date none have been obtained (I. B. Barthelmess, personal communication).

The metabolic signal that the general amino acid control system responds to may be the transfer RNA (tRNA) charging ratio or the amount of uncharged aminoacyl-tRNA (159, 161): mutations in aminoacyl-tRNA synthetases can evoke general derepression of amino acid biosynthetic enzymes despite normal or high pools of amino acids. Derepression upon starvation for an amino acid occurs before the cellular content of the amino acid (189) changes significantly. This suggests that the control system responds, directly or indirectly, to the small, metabolically active pools of amino acids, rather than to the entire cellular content of an amino acid.

Response of arginine biosynthetic enzymes to general amino acid control. Of the arginine enzymes of *S. cerevisiae*, argininosuccinate lyase, acetylornithine:glutamate transacetylase, and the large subunit of CPS-A are controlled solely by the general amino acid control system (159). Argininosuccinate lyase is therefore useful as an example of the system's action. On minimal medium, cells have an enzyme activity of about 2.3 U per mg of protein. Addition of arginine does not repress it further. Cells grown on an amino acid-rich (balanced) medium, however, have activities of about 0.6 U, and arginine or other auxotrophs starved for their supplement (in minimal medium) have activities of about 15 U. Therefore, a 25-fold amplitude of regulation prevails for this enzyme. The mRNA for the enzyme and enzyme activity vary proportionately (162). Argininosuccinate lyase, the transacelylase, and the CPS-A large subunit differ from enzymes also controlled by the arginine-specific ("*ARG80-82*") system in not being repressed by growth in arginine.

The *gcn* class of general amino acid control mutations affect argininosuccinate lyase in several ways. The *gcn3* and *gcn4* mutations impair derepression, and *gcn4* leads to over twofold-lower (basal) activities on minimal medium. Second, the *gcn3* and *gcn4* mutations intensify the effect of rich medium, lowering the argininosuccinate lyase activity another fourfold in comparison with the behavior of the wild-type strain (159). The general amino acid control system controls the large subunit of CPS-A (192) and ornithine carbamoyltransferase in the same way as the lyase (40, 163).

One or more TGACTC sequences have been found in the 5′ nontranscribed regions of arginine genes that have been sequenced, and all show transcriptional regulation by the general amino acid control system (6, 40, 150, 241). The 5′ nontranscribed region of the *ARG3* gene has been analyzed in detail and was shown to have a domain, −363 to −282 with respect to transcriptional initiation, required for response to (general) amino acid starvation. The region contains the more proximal of two TGACTC sequences, and it is distinct from the even more proximal region involved in arginine-specific control (40) (see below).

In *N. crassa*, the general amino acid control system is the only one known to control arginine-biosynthetic gene expression. (The small subunit of CPS-A may be exceptional in having other controlling factors.) This is inferred on the basis of (i) the limited and similar (two- to fourfold) amplitudes of increase of enzyme activity in starved Arg$^-$ mutants (45) and (ii) the observation that a *cpc-1* allele, CD-15, abolished all response of *arg-12* mRNA to arginine starvation (93). The *cpc-1* and *gcn4* mutations of *S. cerevisiae* are similar in reducing enzyme levels seen in minimal medium-grown cultures. It cannot be concluded, however, that the two mutations have the same roles in the system in the two organisms.

The derepression of ornithine carbamoyltransferase and argininosuccinate lyase was studied in detail by Flint et al., who measured the differential rate of accumulation of enzyme activity and enzyme protein during continuous, arginine-limited growth at a suboptimal rate (89). Here, the differential rate of increase was 20-fold that seen in the wild type in minimal medium. This is in contrast to the specific activity achieved by cultures that are allowed to exhaust their supplement and in which protein synthesis stops soon thereafter. Flint's data indicate a quantitative range of derepression governed by general amino acid control comparable to what is seen in *S. cerevisiae*, in which bradytrophs were used to impose the same degree of starvation. A later study showed that, during histidine limitation, *arg-12* transcript levels rose 20-fold, a response similar to that of enzyme activity (93).

Arginine-Specific Control in *S. cerevisiae*

Mutations of the *ARG80-82* system. The arginine-specific control system of *S. cerevisiae* is superimposed upon the general amino acid control system. The specific system adds to the amplitude of regulation of six of the nine biosynthetic enzymes (Tables 3 and 4). The physiological effector is arginine, other amino acids having no direct effect. Muta-

TABLE 3. *trans*-acting regulatory loci[a] affecting arginine metabolism in *S. cerevisiae* and *N. crassa*

System	*S. cerevisiae*	*N. crassa*
General amino acid control	*GCN1* (*aas103*, *ndr1*)	*cpc-1*
	GCN2 (*aas1*, *aas102*, *ndr2*)	
	GCN3 (*aas2*)	
	GCN4 (*aas3*, *aas101*)	
	GCN5 (*aas104*)	
	GCD1 (*tra3*)	
	TRA5	
	Gen^c	
Arginine-specific control (anabolic)	*ARG80*, *-81*, *-82* (*argRI*, *argRII*, *argRIII*)	
	CPA81 (*cpaR*)	
Arginine-specific control (catabolic)	*CAR80*, *-81*, *-82* (*cargRI*, *cargRII*, *cargRIII*)	
	ARG80, *-81*, *-82* (*argRI*, *argRII*, *argRIII*)	
Allantoin degradation control	*DURL*, *DURM*, *DAL80*, *DAL81*	
Nitrogen catabolite control	*GDHCR*?	*nit-2*

[a] Synonyms are given in parentheses.

tions of the system are constitutive for the affected enzymes, which includes ornithine carbamoyltransferase. The first regulatory mutations were found in strains selected for resistance to canavanine, an arginine analog, in an ornithine-containing medium (7). In this medium, ornithine carbamoyltransferase activity and the cellular arginine content are low. Mutants with derepressed ornithine carbamoyltransferase became canavanine resistant. (In retrospect, it seems possible that the effect of ornithine was to displace arginine from the vacuole. Cytosolic arginine initially may have repressed ornithine carbamoyltransferase without being plentiful enough to overcome canavanine inhibition.)

Recessive mutations at three unlinked loci, *ARG80*, *ARG81*, and *ARG82* (originally called *argRI*, *-RII*, and *-RIII* [Table 3]), were found (7, 158). They all imparted similarly derepressed phenotypes, and double mutants were no more extreme than single mutants. All arginine enzymes except CPS-A (both subunits), acetylornithine:glutamate acetyltransferase, and argininosuccinate lyase were affected by the *arg80-82* mutations (249) (Table 4). Arginyl-tRNA synthetase and arg-tRNAs were normal in the mutants (120). An additional, surprising attribute was that the catabolic enzymes, arginase and ornithine transaminase, were uninducible in the *arg80-82* mutant series (248; P. Thuriaux, F. Ramos, J.-M. Wiame, M. Grenson, and J. Bechet, Arch. Int. Physiol. Biochim. **76**:955–956, 1968). This will be considered further in discussion of catabolic enzyme control.

cis-Dominant mutations related to the specific control system have been isolated in two genes. One, *ARG3-O*$^-$, was isolated in a strain selected for its ability to use citrulline as a precursor of carbamoyl phosphate in pyrimidine synthesis (158). The mutant has high levels of ornithine carbamoyltransferase (which catalyzes the phosphorolysis of citrulline), and the enzyme is only weakly repressible by arginine. Double mutants carrying both *ARG3-O* and *arg81* mutations have no more ornithine carbamoyl transferase activity that the *ARG3*$^+$*-O*$^-$ single mutant, suggesting that the site defined by the *cis*-dominant mutation was a target for the normal, *trans*-acting *ARG81* gene product. The *ARG3-O*$^-$ mutation was separable from *ARG3* structural information by recombination (158) and was found later to be a base pair substitution (40) in the 5′ nontranscribed region of the *ARG3* gene. A mutation similar to *ARG3-O*$^-$, *ARG5,6-O*^{c}, has been isolated at the presumed translation-proximal end of the complex *ARG5,6* locus that encodes acetylglutamate kinase and acetylglutamyl-P reductase (125).

The existence of both pleiotropic *trans*-recessive and *cis*-dominant regulatory mutations originally invited comparison with the *lac* system of *E. coli*. As we shall see, however, the system has many unusual features. Among them is the existence of three genes required to repress arginine enzymes. Such a "repressor" would have to be a heterotrimer, a convergence of three independent proteins, or the end product of a metabolic or processing pathway. In addition, it has been suggested that the system has a post-transcriptional component. Finally, the involvement of the arginine-specific system in catabolism, to be discussed later, has led to the discovery of a regulatory cascade (Fig. 6) of which the *ARG80-82* genes are a part.

Molecular aspects of the *ARG80-82* system. Cloning of the *ARG3* (ornithine carbamoyltransferase) gene has allowed dissection of the 5′ elements required for normal regulation (40). As noted previously, the region required for regulation by the general amino acid control system was −364 to −282 base pairs prior to transcription initiation. An independent region between −170 and +22 (the translation start is +22) was required for the specific response to arginine. Indeed, two *ARG3-O*$^-$ mutations were identified with base pair substitutions at positions −80 and −46 in the nontranscribed region. They were 3′ to a good Goldberg-Hogness sequence at −100. Fusions of the *ARG3* 5′ nontranscribed region with the *E. coli galK* gene at codon 1 of *ARG3* displayed regulation characteristic of the *ARG3* gene, indicating that the *ARG3* coding region was not required for its own regulation (40).

The position of constitutive mutations in the 5′ untranscribed region of the *ARG3* gene suggests that arginine-specific control is transcriptional. Yet it had been suggested earlier that a large part of the control was post-transcriptional. Comparison of *ARG3* transcript levels in wild-type cells grown in unsupplemented and arginine-supplemented cells shows little repression of total *ARG3* RNA (10 to 20%) in conditions where the level of ornithine carbamoyltransferase activity is repressed 10-fold (162). The *arg80-82* mutants had approximately three to four times as much *ARG3* RNA as the wild type in arginine-supplemented medium, but ornithine carbamoyltransferase activity was 17-fold greater. The higher level of the RNA in the mutants was attributed in part to its increased stability. An *ARG3-O*$^-$

TABLE 4. Control systems regulating anabolic enzymes of *N. crassa* and *S. cerevisiae*

Enzyme[a]	Control system	
	S. cerevisiae	*N. crassa*
AcGlu synthase	*ARG80-82*, general	General
AcGlu kinase	*ARG80-82*, general	General
AcGlu-P reductase	*ARG80-82*, general	General
AcOrn transaminase	*ARG80-82*, general	General
AcOrn-Glu transacetylase	General	General
Orn carbamoyltransferase	*ARG80-82*, general	General
ASA synthetase	*ARG80-82*, general	General
ASA lyase	General	General
CPS-A small subunit	*CPA81*, general	General, other?
CPS-A large subunit	General	General

[a] Abbreviations as in legend to Fig. 3.

mutant, with 10-fold higher enzyme activity than the wild type when grown on arginine-supplemented medium, also has increased stability of *ARG3* RNA. Later work showed that the *ARG3* mRNA start site was the same in cells grown with and without arginine (40).

These observations suggest that the action of the arginine-specific system is on the translatability of mRNA. In fact, there was good correlation of ornithine carbamoyltransferase activity with *ARG3* polysomal mRNA, but not with nonpolysomal or total *ARG3* mRNA. (Some difficulty prevails in dependably extracting the nonpolysomal mRNA [F. Messenguy, personal communication].) The data imply sequestration of mRNA in conditions of arginine repression; the wild-type *ARG80-82* system might act with arginine to impede exit of mRNA from the nucleus. An *arg80* or *ARG3-O*$^-$ mutation might then cause transfer of mRNA to a polysomal state, where it might be translated and, in fact, be more stable (162).

To reconcile the data on transcript levels with the knowledge of the 5′ sequence of the *ARG3* gene and its mutations, Crabeel et al. propose that the hypothetical repressor (the *ARG80-82* product) may bind to the *ARG3-O*$^+$ region and be carried by RNA polymerase to the transcriptional start (40). The repressor could bind the 5′ end of the transcript, once made, and influence its translatability thereafter. Until the molecular details of arginine-specific regulation are known, however, its post-transcriptional aspect remains controversial.

The *ARG81* gene has been cloned (79) and a large open reading frame of 880 codons (2.64 kilobases) has been sequenced (F. Messenguy, E. Dubois, and F. Deschamps, personal communication). In the earlier work (79) an abundant transcript of 2.8 kilobases was associated with this cloned DNA, but the sequence information showed the open reading frame to be on the opposite strand. The existence of sense and antisense RNAs that these data imply cannot be interpreted at this time. However, the deduced protein sequence of the open reading frame has homologies with the DNA-binding *GAL4* and *PPRI* proteins of *S. cerevisiae*. In addition, the codon usage of the sequence is characteristic of low-abundance proteins, and therefore appropriate for a regulatory protein. The *ARG81* clone does not complement *arg80* or *arg82* mutants.

The *CPA81* system. Two types of mutation affecting regulation of CPS-A in *S. cerevisiae* have been isolated (218). They were selected in a *ura2C* strain, which lacks the pyrimidine-specific CPS-P. Cells of the *ura2C* genotype are prototrophic, owing to carbamoyl phosphate overflow from the arginine pathway (Fig. 4). They acquire a uracil requirement in the presence of arginine, because arginine represses CPS-A. Mutants constitutive for CPS-A were selected. One class is *CPA1-O*$^-$. These are arginine constitutive, closely linked to the *CPA1* locus, and *cis* dominant. The second class consists of *cpa81* mutations. They are *trans* recessive, are unlinked to *CPA1* or *CPA2*, and reduce the arginine repressibility of the small CPS-A subunit, encoded by *CPA1*. The two classes of mutations are quite specific, affecting no other polypeptide of the pathway, although *CPA1-O*$^-$ augments the activity of the large subunit in some conditions (192). The *CPA81* gene is similar, in its effect on CPS-A, to the *ARG80-82* genes in their effect upon their specific target enzymes. Neither directly affects genes addressed by the other. The *CPA81* system, like the *ARG80-82* system, is superimposed upon the general amino acid control system (192).

Cloned probes for *CPA1* and *CPA2* (genes for the small and large subunits) show that the general amino acid control system regulates transcription, while the *CPA81* regulatory system appears to control the small subunit post-transcriptionally (162, 163). The strikingly parallel behavior of the *CPA81* and the *ARG80-82* systems suggests that they are mechanistically similar. A preliminary report, however, states an important difference: one of the *CPA1-O*$^-$ mutations caused a base change in the leader sequence of the mRNA (−98 with respect to translation initiation) for the small CPS-A subunit (A. Feller, M. Werner, F. Messenguy, and A. Piérard, Arch. Int. Physiol. Biochim. **93**:B140, 1985). Further molecular study may test its connection with the postulated post-transcriptional control.

An impasse develops as a result of interaction of the *CPA81* and the general control systems. If cells are starved for histidine or branched-chain amino acids, the large subunit is made at a high rate, but the small subunit is repressed by arginine accumulating disproportionately in the cells (192). The result is a very small rise, or even a decline, in the glutamine-dependent CPS-A activity (163). Under these conditions, the large subunit accumulates in the cytosol in a cryptic form, active only in a complementation assay with the small subunit (192).

Arginine-Specific Control in *N. crassa*

There is no evidence for a system comparable to the *ARG80-82* regulatory circuit in *N. crassa*.

The regulatory behavior of the CPS-A subunits of *N. crassa* in response to arginine (40, 62; R. H. Davis and W. S. Schneider, unpublished experiments) is similar to what is seen in *S. cerevisiae*. Both subunits are nonderepressible in the *cpc-1* mutant in response to arginine starvation. More important, the small subunit is regulated over a very large amplitude, suggesting that a second, arginine-specific system like the *CPA81* system might be operating. However, despite intense efforts (219; Davis, unpublished experiments), no constitutive mutations have been found, even though a simple selection method, similar to that used in *S. cerevisiae* (see above), is available. The different experience with the two organisms may reflect the mitochondrial localization of CPS-A in *N. crassa*. The confinement of CPS-A in mitochondria may allow too limited a leakage of carbamoyl phosphate to the pyrimidine pathway, even those with partially constitutive CPS-A, to allow selection of a CPS-P-less mutant (Fig. 3).

REGULATION OF CATABOLIC ENZYMES

Overview

In this section, the nature and interactions of three major regulatory systems are considered: nitrogen catabolite regulation, inducer exclusion, and arginine-specific regulation (Fig. 6). Nitrogen catabolite regulation is a transcriptional control system that governs many nitrogen-catabolic enzymes and that responds to glutamine as a metabolic signal. Inducer exclusion regulates arginine enzymes by blocking entry of arginine into the cell; a prominent part of this is the inactivation of permeases caused by ammonium. The arginine-specific, catabolic control system is specified in *S. cerevisiae* by regulatory genes which interact with the *ARG80-82* system.

In *S. cerevisiae*, nitrogen catabolite control is a negative system, as far as one can tell, in which glutamine represses the target enzymes (250). An independent role for

ammonium in this system is not excluded (250). In *N. crassa*, the comparable "nitrogen metabolite repression" system (a term used by workers in filamentous fungi) is one in which glutamine opposes a positive regulatory element encoded by the *nit-2* gene (80, 153). This regulator is required for expression of N-catabolic enzymes, including nitrate reductase. Both organisms display inducer exclusion. Finally, *N. crassa* seems to have a simple arginine-specific regulatory system, but it is not yet defined by mutations.

Phenomenologically, *N. crassa* and *S. cerevisiae* are similar in having less catabolic enzyme in minimal medium than in arginine-supplemented medium, and ammonium opposes the augmentation caused by arginine. The complexity of interaction of the systems makes it difficult to resolve its logical elements. For instance, in both fungi, nitrogen starvation leads to increased catabolic enzyme activity, but a large part of this is now known to be due to induction by the arginine being released from the vacuole in these conditions (144, 246). The discussion below introduces the regulatory systems and their action on individual enzymes of *S. cerevisiae*, followed by a briefer treatment of catabolic regulation in *N. crassa*.

Nitrogen Catabolite Control in *S. cerevisiae*

Nitrogen catabolite control is an adaptive mechanism which imparts a hierarchy to nitrogen sources, the ones most economically used generally being used first. Thus maximal elevation of arginase activity is seen only when arginine is present and when ammonium or other preferred nitrogen sources are absent (30, 35, 36, 153, 250).

The major metabolites involved in nitrogen catabolite repression are ammonium, glutamate, and glutamine (30, 250). If ammonium is plentiful, it is converted to glutamate in the nicotinamide adenine dinucleotide phosphate (NADP)-glutamate dehydrogenase reaction; glutamate and another molecule of ammonium are converted to glutamine in the glutamine synthetase reaction. Glutamine (or a closely related derivative) exerts profound negative effects on enzymes devoted to nitrogen assimilation. The effect of glutamine is presumed to be mediated by a key regulatory protein which has yet to be identified. Mutants of the *GDHCR* locus (named for the constitutive state of its NAD-glutamate dehydrogenase activity, but not a structural gene for either glutamate dehydrogenase) are derepressed for many glutamine-controlled enzymes. Wiame et al. (250) hypothesize that the *GDHCR* gene product may be the general, glutamine-binding, nitrogen control aporepressor, working negatively on a variety of structural genes. Ammonium and glutamate, therefore, must be converted to glutamine to exert many of their effects.

It is possible that ammonium may have effects on the nitrogen catabolic control circuit independently of its conversion to glutamine. Not only does ammonium take part directly in inducer exclusion (see below), but it has been suggested that some effects are mediated by the enzyme NADP-glutamate dehydrogenase (which would make this enzyme a regulatory protein) and the *GDHCR* product. The latter question is unresolved, and the reader can explore this issue further by reference to recent reviews (30, 250). The issue is complicated by lack of information on the uptake and intracellular distribution of glutamate, glutamine, and ammonium. This issue will remain controversial until the molecular mechanisms are elucidated.

Inducer Exclusion

A prominent part of "nitrogen catabolite control" is inducer exclusion (35, 36, 72, 100, 101, 153). Recent analyses of the general amino acid permease of *S. cerevisiae* have shown that it turns over rapidly, a process countered by reactivation of inactive molecules and continued de novo synthesis of permease protein. In the presence of ammonium, permease function declines rapidly (36, 100, 101). It is hypothesized that ammonium interacts directly with the inactivation system to promote permease decay, a phenomenon reinforced by glutamine-mediated repression of further permease synthesis (250). This combination of effects allows ammonium to exclude inducers and substrates of catabolic enzymes. Ammonium will therefore appear to be a repressor of catabolic enzymes, despite the fact that it may have no direct effect. Mutations with deranged permease regulation have been isolated; they will certainly contribute powerfully to a molecular analysis of the phenomena of inactivation and synthesis of these proteins (36, 100, 101).

Arginine-Specific Regulatory System of *S. cerevisiae*

Mutations, now known as *CAR80*, *-81*, and *-82*, that affect an arginine-specific regulatory circuit in *S. cerevisiae* (Table 3) were isolated some time ago (72, 248). They caused constitutive activity of arginase and ornithine transaminase and were unlinked to the *CAR1* or *CAR2* gene or to each other. According to the formal model offered originally (248), a *trans*-acting regulator, defined by the *CAR80-82* genes, exerts negative control on arginase and ornithine transaminase. The mutations are very similar in effects, and suggest, as in the case of the *ARG80-82* system, that the *CAR80-82* genes might specify a trimeric protein or the product of a pathway leading to a regulator. The presumed targets of the *CAR80* function are sites (*CAR1-O*$^+$ and *CAR2-O*$^+$) adjacent to the arginase and ornithine transaminase structural genes. The target sites are represented by *cis*-dominant, constitutive *CAR1-O*$^-$ and *CAR2-O*$^-$ mutations (248). The nature of the *cis*-acting site of *CAR1-O*$^+$ will be taken up in detail below.

The inducing effect of arginine is not mediated directly by the *CAR80-82* system, but by other factors, of which the *ARG80-82* system is at least a part. It will be remembered (see "Regulation of Anabolic Enzymes") that *arg80*, *-81*, and *-82* mutations were uninducible for the catabolic enzymes. This demonstrates the requirement for the *ARG80-82* system in catabolic regulation. The observation was extended by finding that *car80*, *-81*, and *-82* mutations are epistatic to *arg80*, *-81*, and *arg82* mutations: the double mutant *arg80 car80* is constitutive and *car80* mutations were, in fact, isolated on that basis (248). The genetic data imply that the action of the *ARG80-82* regulator is to oppose the negative control of the *CAR80-82* regulator on catabolic enzymes (Fig. 6). The level of gene expression at which this takes place has not been defined (see below). A direct, protein-protein interaction was favored initially (72, 248), and the postulated interaction could take place in the cytosol or on the DNA of the catabolic structural genes. The dual role of the *ARG80-82* regulator was further demonstrated by the isolation of an "*argRII*^{d}" mutation, an allele of the *ARG81* gene (78). Unlike the usual *arg81* mutations, *argRII*^{d} is dominant, and strains bearing it display constitutive catabolic enzyme synthesis. Such a strain retains relatively normal anabolic regulation. The phenotype of this strain suggested that the *argRII*^{d} product could bind to, and

inactivate, the *CAR80-82* regulatory protein in an arginine-independent fashion (78). For background literature on the *CAR80-82* system, the reader should see pertinent references (72, 77, 78, 248, 249).

It should be stressed that the models described above are based largely upon physiological behavior of mutations that have been obtained to date. It is clear that many of the uncertainties and controversies about gene action in nitrogen regulation, inducer exclusion, and arginine-specific control can be resolved only by molecular analysis and by recovery of further mutations in functions that this analysis uncovers.

Regulation of Catabolic Enzymes in *S. cerevisiae*

5′ Regulatory sequences of *CAR1* DNA. Recent work on the cloned *CAR1* gene has made it possible to identify sites in the 5′ region that contribute to arginase regulation. The enzyme is repressed by ammonium or glutamine (or both) and the nitrogen catabolite control system. This control is exerted independently of arginine-specific induction by the *ARG80-82*/*CAR80-82* system. We shall go immediately to the nucleotide level and then explore the physiological behavior of the enzyme and its mRNA.

Over 1 kilobase of the 5′ precoding region of the *CAR1* gene has been cloned and mapped by restriction enzymes, and the sequence is known to about nucleotide −600 prior to the start (ATG) of the coding region (126, 212–214). It was shown that the several 5′ mRNA termini (at positions −42 to −49) did not change in relation to induction by arginine- or nitrogen-controlled derepression, or in regulatory mutants (213).

Much of the work on regulation has been done by fusing the 5′ sequences, variously deleted, to the *E. coli lacZ* gene. In one case, this was a protein fusion having only four N-terminal amino acids of the *CAR1* coding sequence (214), while in another sequences 5′ to position −100 were fused with the *CYC1* (cytochrome *c*) promoter and the *lacZ* gene (E. Dubois and R. Cunin, personal communication). The β-galactosidase specified by plasmids carrying these fusions, if they include 1 kilobase of the *CAR1* 5′ sequence, behaves like the arginase specified by the chromosomal copy of the *CAR1* gene of the same cells.

An arginine-constitutive *CAR1-O*$^-$ mutant, *CAR1-O*^{1}, was found to have a single nucleotide change at base pair −153 5′ to the start of translation. This base substitution was related rigorously in gene fusion experiments to the constitutive phenotype (214). The position was in a 10-base sequence also found at −167 in the *CAR2* gene, which is similarly controlled by arginine. Deletion of 13 bases around base pair −153 from the wild-type *CAR1* gene also rendered the expression of the coding sequence constitutive (214).

The most intriguing findings were on the effects of deletions of the 5′ region, measured by using the expression of β-galactosidase directed by *lacZ* protein fusions (215). Deletions 5′ to base −122 yielded a null phenotype, and, indeed, there is a good promoter (TATATAA) sequence centered at base −131 (214). Bal31 deletions starting at the 5′ end of the DNA showed that impaired expression (but with normal arginine and nitrogen regulation) follows 5′ deletions ending between −324 and −219, and no expression at all follows deletions ending at positions −190 and −178. Further deletion (to −149 and −145), which removes the presumed negative control sequence, restored expression, and this expression was constitutive with respect to arginine induction and nitrogen repression. This suggested the existence of a positive control site 5′ to the negative control site at −153. Only when both sites were missing was the promoter, presumed to be the TATATAA sequence centered at −131, able to function freely (deletions to −122 cause a null phenotype) (215). A similar analysis of *CAR1-O*$^-$ 5′ sequences supports this conclusion. In contrast to the findings with the wild-type segment, deletions of the putative positively acting site(s) between −219 and −190 did not impair constitutive expression at all, as if the positive site were not required if the negative site were mutated. As indicated, nitrogen- and arginine-responsive sites in the 5′ region were not resolved.

Another study of this region by Dubois and Cunin (personal communication) manipulated the 5′ *CAR1* sequence with the *CYC1-lacZ* construct, in which no *CAR1* mRNA sequences were included. The intent was to determine whether segments of the 5′ sequence could be identified with the action of the *arg80-82* and *car80-82* mutations. It is easy to presume from the work just discussed that the −153 site identified with a *CAR1-O*$^-$ mutation is in a target for the arginine-specific *CAR80-82* regulatory system. In fact, plasmids bearing the 5′ segment −410 to −100 are completely responsive to arginine-specific regulation. When the segment −190 to −100, including the −153 site, is placed before the *CYC1-lacZ* DNA, expression is low and uninducible by arginine. In keeping with Sumrada and Cooper's results (215), a positively acting site 5′ to base −190 appears to be missing in this fusion. Introduction of the plasmid into *car80*-bearing hosts, however, led to constitutive expression. This too is in keeping with their results (215): without *CAR80* function, the wild-type −153 site could not exert its negative action. It suggests that the −153 site is indeed the target of *CAR80* regulation. (The same constructs introduced into *car81*- and *car82*-bearing hosts did not lead to constitutive expression. These are the only data so far that differentiate the action of the three *car80-82* mutations.)

This work, which continues at a rapid rate, will undoubtedly resolve the following most important outstanding issues. (i) What is the nature of the positively acting site 5′ to the presumed *CAR80* target (around base −153) and the *trans*-acting factor that addresses it? (ii) What is the nature of the nitrogen control site and what is the identity of the *trans*-acting factor that addresses it? Is nitrogen control in fact a negative control system?

Physiological aspects of arginase regulation. There are conflicting reports of the effects of arginine, ammonia, and various repressive nitrogen sources such as serine and asparagine. This reflects strain differences among yeasts used by different workers and different supplementation regimes. However, there is good nitrogen catabolite control, in *car80* mutants, and nitrogen control and arginine control appear to be reasonably additive (77). Further, *arg80-82* mutants have a low, detectable arginase activity that is nitrogen controlled (250). The retention of nitrogen control in both *arg80-82* and *car80-82* mutant classes indicates that nitrogen control is not exerted through these *trans*-acting, arginine-specific regulators. The arginase activity of cells carrying the *cis*-dominant *CAR1-O*$^-$ mutation rises a modest twofold when they are grown in limiting nitrogen sources (77, 250). Because of the very high activity of the *CAR1-O*$^-$ mutant even on ammonium, these data do not clearly indicate whether the muted response to nitrogen limitation is owing to an upper limit of gene expression or to an overlap of the regions responsive to the two regulatory systems, both affected by the *CAR1-O*$^-$ mutation. The latter possibility would be in accord with the phenotypes imparted by certain

of Sumrada and Cooper's deletions of the 5′ region (see above).

Upon starvation for ammonium, wild-type *S. cerevisiae* cells display elevated arginase activity (167). However, if auxotrophs were deprived of arginine and then starved for ammonium, arginase activity did not rise (246). Arginase was still inducible by added arginine or homoarginine (246). Finally, an *arg81* mutant (which remains responsive to nitrogen catabolite control) does not respond to nitrogen starvation (250). All of these data suggest that, upon ammonium starvation of wild-type cells, vacuolar arginine is released into the cytosol and induction takes place. This was proven directly in *N. crassa* (144, 145), the arginase of which does not respond to nitrogen catabolite control (see below).

That nitrogen starvation can act via arginine induction does not preclude nitrogen catabolite control as well, and in fact nitrogen control also prevails, as we have seen. Because nitrogen starvation will stop growth, the long-term effect of this condition must be studied in continuous, nitrogen-limited cultures grown in chemostats (250).

Behavior of *CAR1* mRNA. The level and mechanism of arginase induction, like the nature of nitrogen repression, are controversial (cf. references 162, 213–215). Evidence was first obtained using the enzyme-synthetic capacity of cells in which RNA metabolism was manipulated in vivo as a criterion (13). These studies indicated that the induction of arginase by arginine or homoarginine was due to a stimulation of transcription.

With cloned *CAR1* DNA, Cooper later showed greater amounts of mRNA (estimated at about 12-fold) in wild-type cells grown with arginine as a nitrogen source over those grown with the poor nitrogen source proline (212). This does not clearly resolve arginine induction from relief of nitrogen repression (both aspects may vary in a comparison of arginine- and proline-supported growth). Most of the *CAR1* mRNA was recovered in the polyadenylated fraction (212). On proline medium, constitutive regulatory mutants had more *CAR1* mRNA than did the wild type: fourfold in the case of *car80* and eightfold in the case of *CAR1-O*$^-$. The increase in mRNA was consistent with the higher levels of arginase.

The constitutive mutations responded differently to nitrogen repression, brought about in this work with asparagine as a nitrogen source. *CAR1* mRNA of *car80* was repressed 65-fold by asparagine, while that of *CAR1-O*$^-$ was repressed about 2-fold (212). This indicates again that the nitrogen repression signal does not work through the *CAR80* product. The data for *CAR1-O*$^-$ are compatible with data cited above indicating a twofold nitrogen effect on arginase activity of such mutants.

The data from other groups (126, 162) cannot be reconciled wholly with those of the Cooper laboratory, summarized above. The Brussels group found that the effect of ammonia on *CAR1* mRNA correlated well with effects on enzyme activity (162). However, in the same study, it was concluded that regulation of *CAR1* mRNA by the arginine-specific regulatory system was at least partly post-transcriptional (see below). If this were to be confirmed, it would reflect a remarkable parallel to *ARG3* regulation by the general amino acid and arginine-specific control systems.

A major discrepancy between mRNA and enzyme activity was encountered in a quantitative study of *CAR1* mRNA content and stability (162). Comparing total *CAR1* mRNA with arginase activity, it was found that the enzyme-inducing effects of arginine or of the *car80* mutation (both affecting the arginine-specific system) were not correlated with effects upon mRNA. The small increase (ca. 1.2-fold) could be attributed to greater stability of the mRNA. The greatest mutational effect on mRNA was that of the *CAR1-O*$^-$ mutation, for which a sixfold increase over wild type in ammonium-minimal medium was seen. (This observation is in fact compatible with Sumrada and Cooper's observation on this mutation.) The experiments correlated the larger amount of *CAR1* mRNA, however, with a stabilization of the message (162). The observations on mRNA-enzyme discrepancy are the basis of the postulated post-transcriptional control, in both catabolic and anabolic systems (162). Differences between laboratories regarding the behavior of mRNA (cf. references 162, 212), differences in nitrogen supplementation regimes, and the possibility that nonpolysomal mRNA is not dependably extracted (Messenguy, personal communication) further complicate the issue. The data favoring post-transcriptional regulation can be definitively judged only by further molecular work. It is difficult to reconcile post-transcriptional control with work already done on gene fusions involving *CAR1* 5′ sequences in which the *CAR1* transcript is entirely lacking.

Another type of mutation causes constitutive expression of arginase, and the activity is modulated by the mating type state of the cells. These mutations reflect insertions of Ty1 DNA into the 5′ region of the *CAR1* gene. It was of interest that Ty1 insertions had similar effects whether they were −135 or −700 to −800 base pairs 5′ to the start of translation (126). The *CAR1* transcript sizes were similar. This indicates that transcription can be disturbed by quite long-range influences and that the most 5′ Ty1 insertion imparts constitutivity despite the intactness of the putative positive and negative control sites nearer the coding region.

Ornithine transaminase. For the most part, ornithine transaminase responds to the *trans*-acting mutations *car80-82* and *arg80-82*, much as arginase does (72, 248, 250). It is suggestive, as noted, that the 5′ DNA of *CAR2* (ornithine transaminase) has a 13-mer at −167 in common with the sequence in *CAR1*, in which *CAR1-O*$^-$ mutations lie. Arginine induction of ornithine transaminase is independent of conversion of arginine to ornithine, and homoarginine, a nonmetabolizable analog, can also induce arginase. Ornithine also induces ornithine transaminase somewhat, either because it resembles arginine or because it displaces arginine from the vacuole.

Ornithine transaminase does not to respond to nitrogen catabolite repression. The low ornithine transaminase activity of *arg80* cells (which cannot be induced by arginine) is the same in nitrogen-rich or nitrogen-limited medium, in contrast to arginase activity, which is repressed by good nitrogen sources (250). Similarly, ornithine transaminase activity fails to respond to mutations, such as *gdh1* and *gln1*, that release nitrogen metabolite repression on other enzymes, including arginase. If the enzyme activity of wild-type cells grown in arginine is compared with that of cells grown with arginine and ammonia, activity is greater in the absence of ammonia (72). The difference can be accounted for wholly by inducer exclusion. The level of induction is very closely correlated with the level of the intracellular arginine content over a wide range (72). The data suggest that arginine is the inducer, assuming that there is a direct relationship between the intracellular arginine content and the cytosolic arginine pool. A slight induction of ornithine transaminase by allophanate is described below.

Regulation of urea and proline degradation. Arginine catabolism in *S. cerevisiae* takes advantage of the purine and proline degradative pathways (Fig. 2). The urea produced in

the arginase reaction is degraded by the urea amidohydrolase reactions. This enzyme system, and the allantoin-degrading pathway of which it is a part, is induced by allophanate, the intermediate of urea degradation. The basal levels of allantoin-degrading enzymes are sufficient to produce increased levels of allophanate when allantoin levels increase. The allophanate will then trigger induction of earlier enzymes of the pathway (33). In the case of arginine degradation, urea produced in the arginase reaction will lead to urea amidohydrolase induction and to induction of allantoin-degrading enzymes as well.

Recessive mutations at two loci, *DURL* and *DURM*, render the allantoin pathway uninducible (123). Turoscy and Cooper (223) have isolated pleiotropic *dal81* mutations that render the entire allantoin pathway uninducible. It is not known whether these are allelic to *durL* or *durM* mutations. Cooper's laboratory has also isolated constitutive, recessive *dal80* mutations affecting the same five enzymes (26). The interaction of *dal80* and *dal81* mutations is complex and has not been studied at the molecular level. While the phenotype of the *durL* and *durM* mutants implies positive control, proof that the structural genes are addressed directly by the *DURL* and *DURM* gene products is lacking.

Urea amidohydrolase is subject to nitrogen catabolite control (146). Evidence that this enzyme, like arginase, is repressed not only by glutamine, but also more directly by ammonia, has been adduced by the Brussels group. The reader is referred to the review by Wiame et al. (250) for details.

An unusual feature of ornithine transaminase regulation is its induction by allophanate (and its nonmetabolizable analog oxalurate) (110). It was found that ornithine transaminase was induced in *dur2* mutants, which accumulate allophanate. The *durL* and *durM* mutations, which block induction of urea amidohydrolase by allophanate, also block allophanate induction of ornithine transaminase. Because arginine can induce ornithine transaminase in *dur1* mutants, which cannot make allophanate, the effect of the latter must be secondary. Interestingly, a strain bearing the constitutive, *cis*-dominant *CAR2-O*$^-$ mutation retains some induction (about twofold) by arginine, while the *CAR2-O*$^-$ *dur2* double mutant is fully induced. This implies that the twofold effect of arginine in the single mutant is sequential, via allophanate. Because arginine is an adequate inducer for ornithine transaminase, it is not clear why the enzyme also responds to allophanate.

Finally, the ornithine transaminase reaction, if copious, leads to proline accumulation. Proline induces proline oxidase and pyrroline-5-carboxylate dehydrogenase, both mitochondrial. These degrade proline to glutamate (19, 20). Thus the complete degradation of arginine to glutamate requires sequential induction of the arginine and proline degradative pathways.

Regulation of Catabolic Enzymes in *N. crassa*

Arginase and ornithine transaminase are present at substantial levels in *N. crassa*, even in the absence of external arginine. Arginine induces the enzymes 3- to 10-fold, depending on the enzyme and the medium (60, 86, 95, 237). Arginine is a good inducer of both enzymes; it is an inducer of ornithine transaminase even in arginaseless mutants, which cannot form ornithine (59). Ornithine induces arginase and ornithine transaminase only slightly (59) and may act by displacing arginine from the vacuole. Urease is quite active in *N. crassa*; it participates in purine degradation (51), but its regulation has not been studied. Proline may be an obligatory intermediate in ornithine degradation, as noted previously, but this has not been proved.

A study of the kinetics of ornithine transaminase induction (237) showed that it was induced 30 min after addition of arginine to the medium. Enzyme synthesis, once begun, proceeded at a constant rate despite the continued increase of the cellular arginine content. A second increase in the rate of enzyme accumulation took place 30 min after arginine reached its maximal cellular level. The induction of translatable mRNA (inferred from the capacity to form enzyme in a 90-min period following removal of inducer) took place immediately, however, anticipating the later behavior of enzyme activity. The increase of enzyme activity was dependent upon mRNA and protein syntheses. The results suggest that, for ornithine transaminase, mRNA processing, translation, and protein maturation take a rather long time.

The rate of accumulation of enzyme or translatable mRNA was not correlated with the cellular content of arginine (237). It correlated better with the rate at which arginine entered the cells. This rate, together with the rate of arginine transport into the vacuole, would quickly establish a high, steady-state concentration of arginine in the cytosol. The second phase of induction at maximal arginine levels would be at the time the vacuole, now full, could no longer remove arginine from the cytosol, leading to an even higher level of cytosolic arginine. This interpretation was borne out by the kinetics of loss of enzyme upon transfer of induced cells to minimal medium (237). The enzyme began its decline after a brief lag, with kinetics expected of dilution by growth accompanied by the uninduced rate of enzyme synthesis. This was true despite the presence of a high cellular arginine content. The failure of cellular arginine to sustain induced levels of enzyme suggests that most of the arginine was vacuolar; soon after removing external arginine, the cytosolic arginine concentration fell to a low level.

A recent study (Borkovich, Ph.D. thesis) showed that immunoprecipitable arginase protein increased two- to fourfold upon arginine induction. Curiously, two proteins of similar molecular weight (ca. 42,000 and 38,000, the latter corresponding to the purified protein) were coinduced by arginine and in fact varied somewhat in their ratio in various regulatory mutants and in response to various regulatory stimuli. The 42-kDa protein appears to have little activity and is lost during the purification of the active enzyme. The 42-kDa polypeptide is not only immunologically related to purified arginase, but has an indistiguishable peptide map. Both proteins are missing in all four *aga* (arginaseless) mutants. Both proteins are encoded in separate mRNAs as observed by in vitro translation studies. No clear interpretation of these results can be offered, but they suggest that the *aga* mutations cannot yet be assumed to be structural gene mutations. It might be added that the *ota* mutations have not been analyzed sufficiently to be certain they are structural gene mutations either.

Arginase and ornithine transaminase are not nitrogen metabolite controlled (86). Similar enzyme activities were seen in cultures grown in 10 mM arginine as a sole nitrogen source or in 10 mM arginine plus 25 mM ammonium. Basal enzyme activities were the same in minimal medium with limiting (1 mM) or unlimiting (25 mM) ammonium. A more specific test was made, using a strain carrying the *nit-2* mutation (86). This mutation blocks augmentation of many nitrogen assimilation enzymes, such as nitrate reductase, upon nitrogen starvation (153). The phenotype that it imparts suggests that it has a positive role in enzyme regulation.

Mutants carrying *nit-2* mutations have been correlated with the absence or alteration of a nuclear, DNA-binding protein that is removed from *N. crassa* DNA by glutamine (106). The *nit-2* mutation has no direct effect upon the regulation of arginase and ornithine transaminase (86).

There have been contrary suggestions that arginase activity was in fact controlled by the nitrogen metabolites ammonium and glutamine (226). The observations were accompanied by data on cellular arginine content, which was not grossly affected. However, the steady-state pool was not clearly localized to the cytosol. Later studies (84, 96) acknowledged the importance of knowing the intracellular distribution of arginine, but were unable adequately to specify the instantaneous rate of arginine uptake and sequestration. The difference in the conclusions of Vaca and Mora (226) and Facklam and Marzluf (86) about nitrogen control of arginase may be because the first authors used 1 mM arginine, while the latter used 10 mM as inducer. It is not clear why this difference should create a difference in the regulatory effects of arginine, because transport functions should be saturated with either one. A further point is that Facklam and Marzluf measured arginine transport on a very short-term basis and found that the rate was greatly diminished by ammonium. Thus "nitrogen repression" of arginase here may be exerted by way of inducer exclusion.

A 10-fold increase in the arginine transport rate upon ammonium starvation of the wild type was not seen in *nit-2* mutants (86), indicating again the positive character of the *nit-2*$^+$ action on the permeases responsible (mainly the general amino acid permease). The problematic character of the data of the two groups described above suggests a consideration that must be foremost in experiments of this sort. The cytosolic level of arginine, both a substrate and an inducer of arginase, is determined by relative rates of arginine uptake, synthesis (if it occurs), sequestration into the vacuole, and the use of arginine in protein synthesis, i.e., growth. It is virtually impossible to know what the cytosolic levels are unless they are measured directly, with an isotopic method.

In a study focussed on the effect of glutamine on arginine mobilization from the vacuole, Legerton and Weiss (145) demonstrated that the metabolic signal for mobilization was a glutamine deficiency. Thus, upon nitrogen starvation, arginine exits the vacuole to the cytosol and induces arginase. The authors suggest that arginase and ornithine transaminase are removed from *nit-2*$^+$ control (86) because arginine is a prominent nitrogen reserve in vacuoles. Nitrogen starvation could therefore lead to mobilization and use of arginine without the need for *nit-2*$^+$-induced gene expression. Gene products dependent upon *nit-2*$^+$ could therefore be synthesized readily from the nitrogen metabolites that arginine would provide more immediately. Unfortunately, the role of the *nit-2*$^+$ product upon arginine mobilization was not specifically tested.

BIOCHEMICAL INTEGRATION OF ARGININE METABOLISM

Anabolic Flux to Arginine

Introduction. The metabolic rates of many reactions of the arginine pathway of *N. crassa* are known, and our understanding is enhanced by knowledge of compartmentation of small molecules. In fact, even rates of transport across intracellular membranes have been measured. By contrast, less about determination of flux is known in the case of *S. cerevisiae*. Therefore, this discussion will concentrate mainly upon *N. crassa* until adaptive mechanisms in the two fungi are compared.

In a theory of metabolic flux that owes much to the contemplation of the arginine pathway of *N. crassa*, Kacser and Burns (133) emphasize the buffering capacity of biochemical pathways. Because enzymes are usually not saturated, perturbations of a given enzyme parameter (e.g., V_{max}) are followed by adjustment of metabolite pools in the pathway that compensate at least partially for the change. The result is that transient effects of small changes of a given step upon the overall flux are rarely proportional. Instead, the quantitative relation can be expressed as a sensitivity coefficient between 0 and 1.0, and the coefficients of the steps of all enzymes of the pathway sum to 1.0. While the initial enzyme of a pathway may have a high sensitivity coefficient, and while drastic impairments of an enzyme may render it almost wholly rate limiting at steady state, many enzymes can vary greatly without severe impact on pathway flux (4, 91). Cases in point are the *arg-12*^{s} mutation, causing a 95% decrease in ornithine carbamoyltransferase activity, and certain partial revertants of *arg-10* mutations, which do not lead to severe growth impairments (4, 46, 132). Although flux is indeed diminished, largely by failure to maintain the vacuolar pool, and although derepression of enzymes is required for the growth actually seen, such mutations are remarkable for their minor impact on growth. Savageau has presented a similar view of biochemical systems (204) and has extended it by analyzing existing regulatory systems with respect to optimal design considerations.

An early study showed that the activity of the last three enzymes of the pathway were at their fully repressed level in minimal medium (4). This conclusion was later extended to most other enzymes (45, 114a). Only when the cellular arginine content dropped to one-quarter the level seen in minimal medium-grown mycelia did derepression begin (4). Curiously, feedback inhibition appeared not to be called upon in minimal medium (91; I. Goodman and R. L. Weiss, J. Biol. Chem., in press). Mutations (*sup-3*) to feedback resistance of acetylglutamate kinase had no effect upon the flux of [^{14}C]glutamate into the pathway in cells grown in minimal medium (96). This meant that little feedback inhibition was exerted by the arginine content of wild-type cells grown in minimal medium. These observations suggest that, in relation to the state of minimal medium-grown cells, feedback inhibition is the adaptive response of the pathway to increases in arginine concentration, while derepression is the adaptive response to depletion of arginine (91).

A study by Hilger et al. (112) in *S. cerevisiae* took a similar approach to the question of sensitivity of the system to enzyme depletion. With tetraploids, they were able to vary the wild-type/mutant allele ratio from 0:4 to 4:0 for four enzymes. They found that limitation of the wild-type alleles of the *ARG5,6* locus, imposing a deficiency for acetylglutamate kinase activity, and of *CPA1*, imposing a deficiency for the small subunit of CPS-A, led to increased activity per wild-type allele, starting at about 2 wild-type to 2 mutant alleles. The derepression of these gene products, which are the "entry points" of the pathway, was accompanied by derepression of the other enzymes. The latter was a systemic response to the limitation of the arginine pool imposed by the heterozygous locus. In contrast, a similar series for *ARG3* (ornithine carbamoyltransferase) and *ARG4* (argininosuccinate lyase) showed no such effect, either on the arginine pool or on derepression of enzymes in the wild type/mutant range of 1:3 to 4:0. The results demonstrate differential sensitivity

of the pathway to limitations of different enzymes, a conclusion compatible with the Kacser-Burns theory. Hilger et al. (112) stress the importance of the entry points of the pathway having a large role (high sensitivity coefficients) in the determination of flux, whereas Kacser would emphasize the formal distribution of sensitivities over many enzymes (4, 133).

At cellular arginine contents approximating those found in *N. crassa* grown in minimal medium, neither feedback inhibition nor derepression prevails. There are several other parameters that could respond to fluctuations of the arginine and ornithine pools: (i) reverse reaction of argininosuccinate lyase and acetylornithine:glutamate acetyltransferase; (ii) "overflow" catabolism of arginine or ornithine; and (iii) vacuolar uptake and release of these amino acids. Little is known about the first of these possibilities, except that growth in arginine leads to accumulation of some argininosuccinate through the reverse reaction, and this is likely to have some role in buffering cytosolic arginine concentrations. The same may be true of ornithine in the acetyltransferase reaction. The other mechanisms are best considered together.

Cellular distribution and metabolic fate of arginine. In wild-type cells grown in minimal medium, substantial catabolic enzyme activity coexists with high concentrations of their substrates. This was initially a paradox in view of the observation that no arginine was catabolized during growth of *N. crassa* (51, 208). The early work on the vacuole of *N. crassa*, however, showed that <10% (206, 235), later reduced to about 1% (135), of the cellular arginine was cytosolic. The methodology used in these and other studies allowed determination of many of the dynamic parameters of pool distribution and the metabolic fates of intermediates.

The method used to find cytosolic amino acid pool sizes was based on isotope dilution. The rationale was to determine the isotopic dilution of a compound, added externally at high specific radioactivity, as it entered the cell, mixed with the cytosolic fraction of that compound, and was used in metabolic reactions. Thus Subramanian et al. (208) showed that a tracer level of [^{14}C]arginine, added to cells growing in minimal medium, all entered cells within 2 min. It found its way into protein within 10 s, with much less dilution than would have occurred if all of the cellular arginine were cytosolic and accessible to protein synthesis. In this and other studies (15, 16, 135, 236), the specific activity of the substrate of a reaction in vivo (e.g., arginine in protein synthesis) was determined by dividing the counts appearing in the product of that reaction by the nanomoles of the substrate entering product in the same interval. The denominator was calculated independently from the rate of synthesis of the product (and its derivatives) required to maintain the steady state during exponential growth.

Three ways of calculating the cytosolic pool of arginine have been used (135). One was to determine the time needed to replace the cytosolic arginine pool (the precursor of protein arginine) one time as the pool approached constant specific radioactivity. This was done by extrapolating the linear phase of entry of counts into protein to the x axis of the time curve. With the rate of entry of arginine into protein being known, the amount of arginine which entered protein in the interval between time zero and the x intercept could be calculated. This figure was the amount of arginine in the cytosol at steady state. The second method was to determine the dilution of added arginine by the cytosolic pool as the mixture was fixed into protein in the first (10- to 20-s) interval. The third method was to compare the specific radioactivity of arginine being used as a protein precursor with the total arginine of the cell. If the former is 50-fold more radioactive than the latter, then 1/50 of the arginine in the cell is cytosolic. The best estimate of the amount of arginine in the cytosol is about 1% of the total arginine of the cell (135). An experiment in which [^{14}C]arginine is used by wild-type cells and an interpretative diagram are presented in Fig. 7.

[^{14}C]arginine, when taken up by *N. crassa*, is not only used for protein synthesis, but is also sequestered in the vacuole. There it persists, exchanging with the arginine of the cytosol. At steady state (after all [^{14}C]arginine has been taken up from the medium), the exchange rate across the vacuolar membrane could be calculated (15, 135). The rate calculations suggested that about one-third to one-half of the arginine that was used for protein synthesis had passed through the vacuole (208). In terms of net amounts, *N. crassa*, grown in minimal medium, allocates about 89% of its biosynthetic arginine to protein synthesis and 11% to maintaining the vacuolar pool.

Finally, the isotopic data showed that arginine taken up from the medium, arginine emerging from the vacuole, and arginine that was formed in the argininosuccinate lyase reaction were indistiguishable kinetically. This put to rest an issue raised by work in the 1950s (37, 38), which suggested that biosynthetic (endogenous) and exogenously added amino acids had different relationships to protein synthesis.

If the cytosolic pool of arginine were 1% of the cellular arginine, and if cytosolic water were 80% of cell water, the concentration of arginine in the cytosol would be about 0.1 mM. At this level, the rate of urea formation by a Michaelian arginase (K_m [arg] = ca. 20 mM) would be detectable over a long period of growth. As noted above, no arginine catabolism was seen in such cultures, owing to sigmoid substrate kinetics and severely suboptimal conditions in vivo for the enzyme.

In summary, the steady state of the arginine pools in cultures grown in minimal medium is maintained by distribution of arginine to the vacuole and to protein synthesis, leaving little in the cytosol exposed to arginase and arginyl-tRNA synthetase. The K_m of arginase, ca. 20 mM (Borkovich, Ph.D. thesis), is much higher than that of arginyl tRNA synthetase (2×10^{-5} M) (173). This, combined with other conditions relating to arginase activity, forces all arginine toward storage or protein synthesis. In view of the full repression of the enzymes, and the lack of feedback inhibition in these conditions, the steady-state arginine pool is maintained by enzyme design and arginine compartmentation. This will become clearer when we later consider the transition to catabolic conditions.

Cellular distribution and metabolic fate of ornithine. Ornithine is made in mitochondria, where it is used by ornithine carbamoyltransferase. To reach ornithine transaminase, ornithine decarboxylase, and the vacuole, ornithine must pass across the mitochondrial membrane. (Ornithine is also an intermediate of the catabolic pathway in the cytosol, and the management of ornithine during the transition to the catabolic state involves unusual regulatory phenomena. This will be discussed in a later section.)

According to a tracer study, about 0.3% of the ornithine in cells growing in minimal medium is cytosolic (135). This was inferred from the isotopic dilution of a very small amount of [^{14}C]ornithine as it was used for putrescine and spermidine synthesis. About 1% of cellular ornithine is mitochondrial. This was inferred from the further dilution of labeled

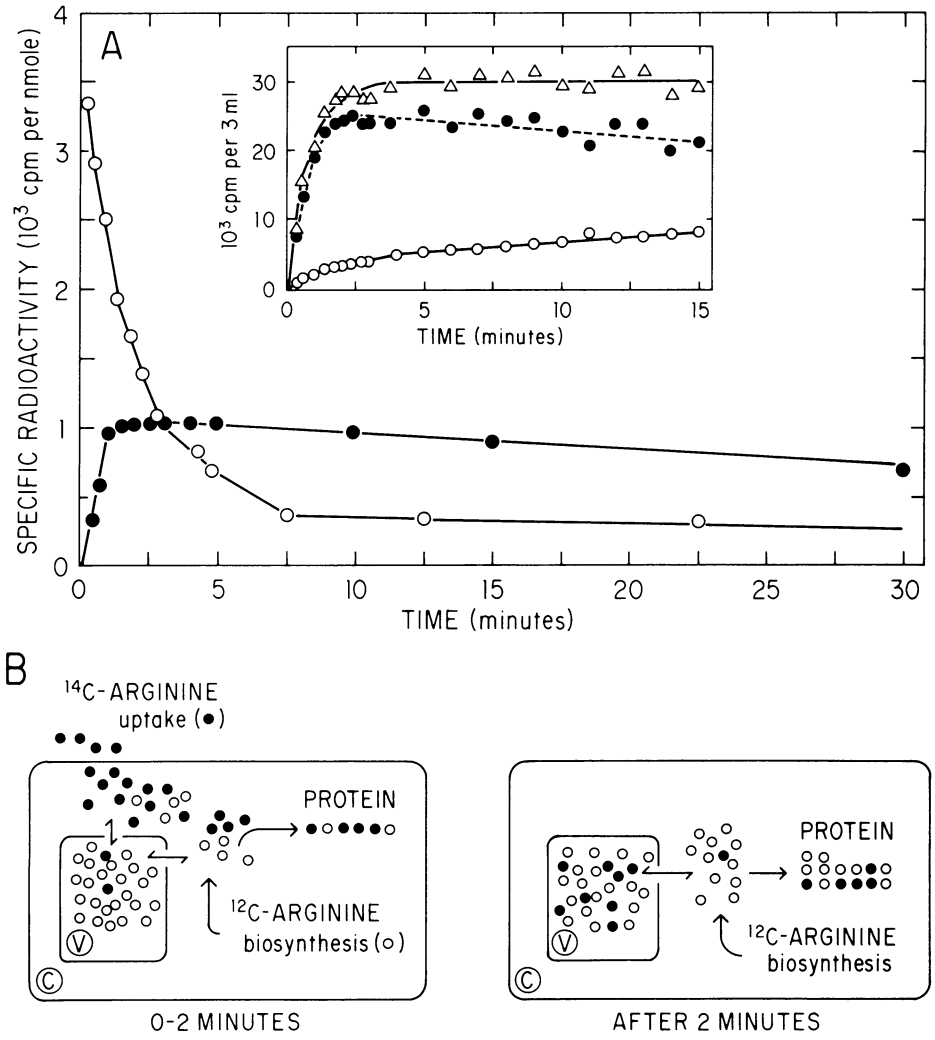

FIG. 7. Fate of a small pulse of [^{14}C]arginine added at time zero to *N. crassa* growing in minimal medium (from reference 44). (A) (Main axes) Specific radioactivity of the arginine being used as a protein precursor (open circles) and of the total, trichloroacetic acid-soluble arginine (closed circles) of the same cells. (Inset) Raw data from the same experiment, showing radioactivity entering the cell (triangles), protein arginine (open circles), and extractable, soluble arginine (closed circles). (B) Interpretation of the specific radioactivity data of (A). Early in the experiment, [^{14}C]ornithine (closed circles) is used selectively for protein synthesis over [^{12}C]arginine (open circles) confined in the vacuole; the radioactive arginine mixes only with the very small, endogenous, cytosolic pool. At later times, the remaining radioactive arginine is confined to the vacuole and is somewhat excluded as a protein precursor. See references 57 and 208 for full discussion of these phenomena.

ornithine as it passed from the cytosol to become a substrate of the ornithine carbamoyltransferase reaction. By elimination, the rest (over 98%) was presumed to be in the vacuole, and this was supported by Weiss's earliest work on this organelle (235).

The determination of ornithine distribution in vivo was based on samples taken in the first 0.5 min or so after addition of [^{14}C]ornithine to a minimal medium-grown culture. A quasi-steady state was reached during ornithine uptake (0 to 2 min), during which it was possible to calculate the exchange across the mitochondrial membrane (15, 135). The rate at which ornithine entered mitochondria was approximately equal to its rate of synthesis inside the mitochondrion. This implied that the rate of exit was even faster, because ornithine is diverted in the cytosol to the vacuole and the two cytosolic enzyme reactions. The exchange rate across the vacuolar membrane was also rapid, the exit and entry rates being 75 to 85% of the rate that ornithine was used in the ornithine carbamoyltransferase reaction (135).

The exchange of ornithine across the mitochondrial and vacuolar membranes suggests that these processes might exert metabolic control. In fact, as we shall see below, both exchanges are profoundly altered during catabolism. At the anabolic steady state, however, the cytosolic ornithine concentration is determined by biosynthetic enzymes which are not feedback inhibited, but fully repressed, together with the net utilization and exchange rates noted above. What would happen if ornithine concentrations in the cytosol should rise unduly? First, ornithine can be taken into the vacuole. (This can be directly shown after addition of ornithine to cells.) Second, the ornithine transaminase reaction, in contrast to the arginase reaction, actually functions in anabolic conditions as an "overflow valve." Approximately 5 to 10% of ornithine biosynthesized is lost through this Michaelean reaction at steady state (15, 135). The fate of the product is

proline, and therefore the ornithine "lost" is actually captured in a biosynthetic end product (135). (This may be one of the reasons that proline has been retained as an intermediate in arginine catabolism, at least in *S. cerevisiae*.) The reaction is probably sensitive to excess or depletion of the cytosolic ornithine pool, because the K_m for ornithine (ca. 2 mM) is over 10-fold the cytosolic ornithine concentration in cells grown in minimal medium (30 to 100 μM) (66).

Ornithine entering the cell from the medium may have a special relationship to the ornithine transaminase reaction. During the brief period of uptake, isotopic ornithine was used much more rapidly than after it entered, as though the transaminase (or a fraction of it) was juxtaposed to the membrane transport system (15, 135). Such a relationship had often been suspected (50, 230), but, as in this instance, no direct proof was obtained.

Ornithine decarboxylase does not require much ornithine to maintain cellular polyamines (187). The amount of ornithine used for polyamine synthesis is approximately the same as the amount catabolized (135). The K_m of ornithine decarboxylase is approximately 0.2 mM (85), and the enzyme is highly regulated by synthesis and inactivation (58). It is therefore able to respond quickly to alterations of ornithine concentration (187), and it is fully saturated in catabolic conditions.

To summarize, the normal allocation of ornithine is 8% to the ornithine decarboxylase reaction, 11% to the transaminase reaction, 74% to the ornithine carbamoyltransferase reaction, and 7% to the vacuole (15, 57). The allocation takes place while ornithine exchanges rapidly across the mitochondrial and vacuolar membranes. High concentrations of ornithine are maintained, however, in the mitochondrion and in the vacuole. The implication of the high levels of ornithine in the mitochondrion and the loss of ornithine to catabolism is that rates of citrulline synthesis are more sensitive to variation in the rate of carbamoyl phosphate synthesis than to variation in the rate of ornithine synthesis.

Onset of Catabolism

N. crassa. A number of processes ensue upon addition of arginine to a culture of *N. crassa* growing in minimal medium (Fig. 8A). First, arginine rapidly enters the cell and raises the cytosolic arginine concentration (236). Urea formation (measurable in *ure-1* strains) begins immediately. Arginine also enters the vacuole and displaces ornithine (16). A high and constant rate of ornithine consumption from the cytosol by ornithine transaminase therefore begins. This is sustained thereafter by ornithine from the arginase reaction (16). Thus, within seconds after arginine is added, a functioning catabolic pathway is established, complete to glutamate-γ-semialdehyde and proline (66) (Table 5). After 1 h, the cellular arginine content has risen by 10-fold, 20% of which is cytosolic (236). The cytosolic concentration of arginine is estimated at 15 mM. The ornithine content of cells drops by this time to 45% of normal, but all of it is cytosolic. The cytosolic ornithine concentration is about 5 mM (16). The concentrations of both catabolic substrates, then, equal or exceed the K_ms of the enzymes that use them. The onset of catabolism occurs well before catabolic enzymes are induced (237, 239).

The second event is the entry of arginine into the mitochondria. There, it feedback inhibits acetylglutamate kinase and acetylglutamate synthase in less than 3 min after addition of arginine (16, 96). The result is that ornithine synthesis ceases in mitochondria, and further citrulline synthesis takes place at the expense of ornithine that enters from the cytosol (15, 16, 96). Tracer studies show that ornithine remains available there in high concentration as a product of the arginase reaction, but that rather little enters mitochondria (15, 16). Because arginine does not feedback inhibit CPS-A or ornithine carbamoyltransferase activity, we must ask why mitochondrial ornithine does not flood the mitochondria and drive an active ornithine cycle.

The answer to this question is that the high level of arginine in the cell inhibits passage of ornithine into mitochondria. The net effect of blocking intramitrochondrial synthesis of ornithine and reducing transport of ornithine from the cytosol is an immediate reduction of citrulline synthesis to 25% of the rate prior to arginine addition (15). This rate (determined with isotopic methods) diminishes slowly with time (15). Only after 8 h following arginine addition, or more than two mass doublings, does citrulline synthesis become undetectable.

Further study of these phenomena was done on the *arg-1* strain. This mutant lacks argininosuccinate synthetase and therefore accumulates citrulline when it is starved for arginine. The control of citrulline synthesis could therefore be analyzed more directly than in the wild type. Mycelia of strains carrying the *arg-1* mutation were grown in arginine, washed, and transferred to arginine-free medium. Derepression of CPS-A and relief of feedback inhibition led within 2.5 h to a constant and high rate of citrulline accumulation. Addition of arginine during this phase led to immediate reduction of citrulline synthesis to about 5 to 10% of the prior rate (61). This is a somewhat greater proportional effect than in wild-type cells (see above), but it is very similar in absolute terms.

To demonstrate that the effect of arginine was not directly on CPS-A or ornithine carbamoyltransferase, a double mutant strain carrying both the *arg-1* mutation and *sup-3*, a mutation conferring feedback resistance upon acetylglutamate kinase, was used. This strain was immune to the effect of arginine upon citrulline synthesis (61). This showed that neither CPS-A nor ornithine carbamoyltransferase was seriously inhibited by arginine in vivo. The important difference between the *arg-1* and the *arg-1 sup-3* strains was that ornithine continued to be made in the mitochondria of the double mutant. The data suggested that arginine, when added to the *arg-1* strain, blocked the entry of cytosolic ornithine into mitochondria as well as feedback inhibiting the synthesis of ornithine within mitochondria. Other data, using other amino acids, showed that arginine was probably competing with ornithine for a basic amino acid transport function in the mitochondrial membrane. In vitro tests to define this function were unsuccessful (T. Paulus and R. H. Davis, unpublished experiments).

The above experiments illuminate the control of ornithine synthesis and utilization, but they pose a serious economic question about the control of carbamoyl phosphate synthesis. If arginine does not inhibit CPS-A, copious accumulation of carbamoyl phosphate is expected when mitochondria are deprived of ornithine. Three possibilities might mitigate this expectation: (i) carbamoyl phosphate might accumulate within mitochondria to a low level defined by a balance of synthesis and degradation; (ii) it might accumulate within mitochondria and inhibit its own synthesis; or (iii) it might accumulate in mitochondria, spill into the cytoplasm, and be used in the aspartate carbamoyltransferase reaction. The last possibility was directly tested by using an *arg-1* strain that also carried mutations of pyrimidine enzymes. The latter

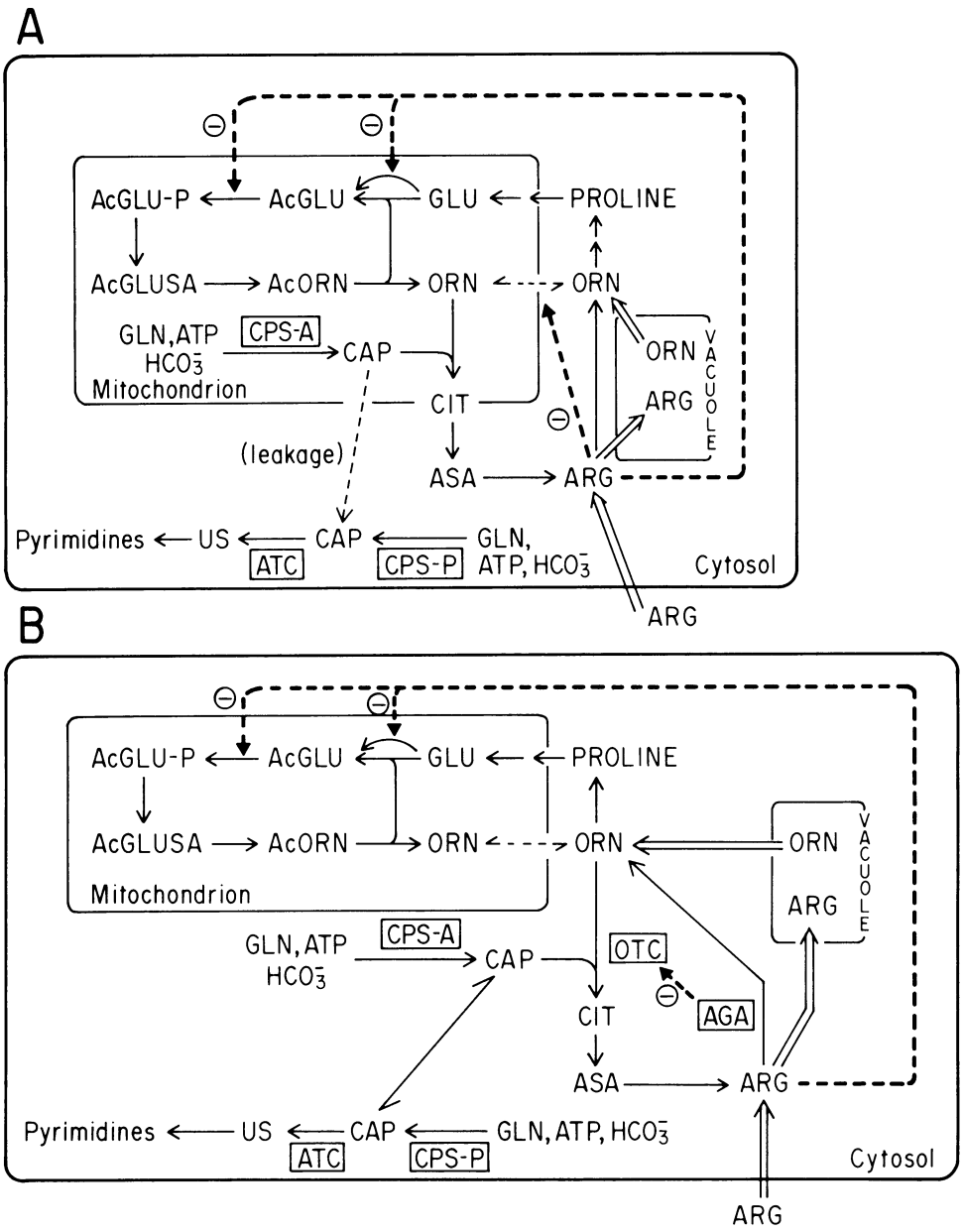

FIG. 8. Short-term regulation of arginine metabolism during the onset of catabolism in *N. crassa* (A) and *S. cerevisiae* (B). The long, heavy broken lines indicate feedback inhibition by arginine of acetylglutamate synthase and acetylglutamate kinase in both organisms. In addition, alternative mechanisms, reflecting different enzyme localizations, block entry of catabolically derived ornithine into the anabolic reactions. In *N. crassa*, arginine blocks entry of ornithine into the mitochondrion. In *S. cerevisiae*, arginine induces arginase, which aggregates with ornithine carbamoyltransferase and inhibits it. Abbreviations: AGA, arginase, ARG, arginine; ASA, argininosuccinate; CAP, carbamoyl-P; CIT, citrulline; GLN, glutamine; OTC, ornithine carbamoyltransferase; US, ureidosuccinate. Other abbreviations are given in the legend to Fig. 3.

were chosen so that carbamoyl phosphate emerging from mitochondria could be trapped in the aspartate carbamoyltransferase reaction as ureidosuccinate. When arginine was added to cells accumulating citrulline, ureidosuccinate began to accumulate, but only at 5% of the prior rate of citrulline synthesis (Davis and Ristow, unpublished experiments). Thus carbamoyl phosphate is not copiously diverted to the pyrimidine pathway in these transient conditions when it is no longer used within mitochondria. Direct tests show that the tiny carbamoyl phosphate pool of 0.01 nmol per mg of protein rises to 0.4 nmol/mg (presumably confined in mitochondria) when arginine is added and then falls again to the previous level after about 1 h.

If cells of the *arg-12* mutant, lacking ornithine carbamoyltransferase, are starved, they accumulate carbamoyl phosphate up to a limit of 0.8 nmol per mg of protein. This is over 20-fold the level found in *arg-1* mycelia in which carbamoyl phosphate is being used for citrulline synthesis. However, it is enough carbamoyl phosphate for only 12 s of citrulline synthesis, a very small portion of the carbamoyl phosphate-forming potential. It appears to be self-limited by reduction of synthesis, increase of degradation, or both, quite apart

TABLE 5. Rates of enzyme reactions in vivo before and after addition of 1 mM arginine to cultures grown in minimal medium (from reference 66)

Enzyme	Reaction (nmol/min per mg dry weight) at interval analyzed:		
	Prior to arginine addition	0-60 min after arginine addition	Steady state in arginine
Ornithine carbamoyltransferase	0.86	0.17	0
Ornithine decarboxylase	0.09	0.13	0.11
Ornithine transaminase	0.13	0.61	1.07
Arginase	0	0.96	1.30

from any effect of arginine (Davis and Ristow, unpublished experiments). The mitochondrial membrane presumably confines carbamoyl phosphate and establishes the limit on its accumulation. However, if arginine is added to the arginine-starved *arg-12* mutant, the accumulated carbamoyl phosphate is reduced to low levels in about 1 h. The mechanism of this effect is unknown and is currently being explored.

The responses of *N. crassa* to arginine addition can be summarized as follows. (i) The vacuole allows a rapid onset of catabolism by discharging ornithine to the cytosol and by being somewhat slower to take up arginine than it enters from the medium. (ii) Feedback inhibition of intramitochondrial ornithine synthesis is rapid. (iii) The mitochondrial membrane, in the presence of arginine, minimizes a futile ornithine cycle by blocking access of cytosolic ornithine to ornithine carbamoyltransferase. (iv) Carbamoyl phosphate accumulation and its diversion to the pyrimidine pathway are limited by unknown mechanisms. (v) Little induction of arginase (239) or ornithine transaminase (237) occurs in the first hour. All of the immediate metabolic responses to arginine take place in the absence of protein synthesis and, thus, do not require gene expression.

S. cerevisiae. When arginine is added to *S. cerevisiae* cells growing in minimal medium, arginase and ornithine transaminase are quickly induced (13). This is different from the sluggish induction of the enzymes of *N. crassa*. Both arginine and ornithine become plentiful in the cytosol. In *S. cerevisiae* an unusual mechanism leads to inhibition of ornithine carbamoyltransferase at the onset of catabolism (Fig. 8B).

In ornithine carbamoyltransferase assays of permeabilized cells, the activity declined rapidly after arginine addition, with the kinetics of arginase induction (8). Ornithine carbamoyltransferase activity was restored upon extraction of cells. The phenomenon, reconstructed in vitro, involves the specific aggregation of one molecule each of ornithine carbamoyltransferase and arginase in the presence of arginine and ornithine. The inhibition of ornithine carbamoyltransferase is complete at high levels of arginine; arginase activity is not inhibited (164, 165, 248). The inhibition of ornithine carbamoyltransferase by arginase is possible because, in *S. cerevisiae*, both enzymes are cytosolic. Clearly, *N. crassa* could not use this mechanism, nor could *S. cerevisiae* limit the cycling of ornithine by inhibiting its transport across the mitochondrial membrane.

Inhibition of ornithine carbamoyltransferase by arginine is an intensification of substrate inhibition by ornithine. Originally, an isosteric site on ornithine carbamoyltransferase, distinct from the substrate binding site, was postulated, owing to the ability of acetylation and certain mutations in the *ARG3* gene to nullify substrate inhibition without disturbing catalytic activity (164). More recent biophysical studies, which confirm the basic phenomenology, reveal no isosteric site for ornithine by several sensitive criteria; the site involved in substrate inhibition is in all probability the active site (82a). The 1:1 arginase-ornithine carbamoyltransferase complex postulated previously on the basis of gel filtration and enzyme inhibition studies has been visualized by transmission electron microscopy. The complex is indeed a face-to-face 1:1 stoichiometric aggregate of the two trimeric enzymes, although the exact orientations of the two molecules in the aggregate is not known (E. Eisenstein, L. T. Duong, R. L. Ornberg, J. C. Osborne, Jr., and P. Hensley, submitted for publication).

The term "epiprotein" (and in this case, "epiarginase") has been coined to refer to proteins having regulatory, inhibitory action through stoichiometric binding (248). It is similar to the term "antizyme," coined more recently for an ornithine decarboxylase inhibitor of procaryotic and eucaryotic cells (21).

The physiological significance of the epiarginase phenomenon can be inferred: upon the appearance of high levels of arginine in the cytosol, catabolism will begin, aided by induction of arginase. The ensuing inhibition of ornithine carbamoyltransferase would block a futile ornithine cycle. (As in *N. crassa*, CPS-A is not feedback inhibited by arginine in vitro [248].) The presumed role of arginase-ornithine carbamoyltransferase aggregation still requires confirmation by showing (i) that addition of arginine inhibits citrulline synthesis in vivo and (ii) that mutations rendering ornithine carbamoyltransferase insensitive to arginase inhibition cause continued cycling of ornithine through ornithine carbamoyltransferase after arginine is added to cells. It is also important to determine what happens to carbamoyl phosphate synthesis or accumulation after arginine addition. It may be that *S. cerevisiae* and *N. crassa* are similar in their control of carbamoyl phosphate accumulation or that different mechanisms, correlated with the different locations of CPS-A, have evolved in the two organisms.

A systematic study of other yeasts shows that all species of *Saccharomyces* and a few others have the epiarginase mechanism of ornithine carbamoyltransferase inhibition (225, 228). None were obligate aerobes. The mechanism correlates exactly with the cytosolic location of ornithine carbamoyltransferase and CPS-A and for the most part with substrate inhibition of ornithine carbamoyltransferase. In addition, inhibitory arginases were trimeric, as opposed to hexameric (190).

The remaining features of the onset of catabolism of *S. cerevisiae* may be similar to those of *N. crassa*, although little direct evidence is available. The two organisms differ, however, in the role of enzyme regulation. In contrast to *N. crassa*, complex regulatory responses are prominent in *S. cerevisiae*, especially the induction of ornithine transaminase and arginase (in its catalytic and its epiarginase roles). It should be remembered that arginine exerts its regulatory role through the *ARG80-82* system. This system overcomes the negative action of the *CAR80-82* system on the catabolic enzymes and represses many of the anabolic enzyme activities. The most telling point of difference between the organisms is that there is no counterpart of the *ARG80-82/CAR80-82* system in *N. crassa*, although arginine does induce its catabolic enzymes.

Catabolic Steady State

During steady-state growth of *N. crassa* in the presence of arginine and ammonium, more arginine is catabolized than is

used for protein synthesis (59). It is not known with certainty that all arginine is catabolized through proline, as it is in *S. cerevisiae*. At steady state, the arginine pool is threefold, and the ornithine pool is one-third, those found in cells grown in minimal medium. Arginase and ornithine transaminase are induced three- to fivefold, and catabolism is 35 to 40% faster than immediately after arginine is added (66). Despite the fact that ornithine appears via catabolism at a greater rate than it is biosynthesized during growth in minimal medium, it is neither stored nor cycled. Instead, it is channelled largely into the ornithine transaminase reaction, owing to the arginine-blocked entry of ornithine into vacuoles and mitochondria. All ornithine arising from catabolism at steady state can be accounted for roughly in reactions other than ornithine carbamoyltransferase (Table 5).

Three doublings (8 h) are required to achieve this state. This is a time consistent with the dilution of preexisting CPS-A to its repressed level of one-fourth to one-tenth that seen in minimal medium-grown cells (15, 45, 62). A direct test of citrulline accumulation in an *arg-1* (argininosuccinate synthetase) mutant, grown in arginine at steady state, reveals that it takes place at approximately 1% of the rate citrulline is made by wild type on minimal medium. One question that can be posed is how much the reduced citrulline synthetic rate is due to CPS-A repression, and how much is due to ornithine exclusion from the mitochondrion.

To answer this question, the *arg-1 sup-3* double mutant was grown in steady state on arginine and ammonia. Here, the citrulline accumulation was about 10% of the rate that wild type, grown in minimal medium, synthesizes citrulline (Davis and Ristow, manuscript in preparation). The 10-fold-greater rate of citrulline synthesis in the feedback-insensitive strain reflects the availability of ornithine within the mitochondria. Therefore, the factor by which CPS-A repression reduces citrulline synthesis must be approximately 10-fold also, in very rough agreement with the reduction in the in vitro activity of the enzyme.

The prediction that CPS-A could limit arginine synthesis in arginine-containing media was also tested by Goodman and Weiss, using the *sup-3* mutant (Goodman and Weiss, unpublished experiments). Even after prolonged growth of this strain in arginine, the radioactivity of [^{14}C]glutamate enters the pathway at half the uninhibited rate, whereas almost none enters the pathway in arginine-grown wild type. The partial inhibition of entry of glutamate by arginine is consistent with the residual sensitivity of the mutant kinase to arginine. It is not possible from the data to calculate how much of the label that entered the pathway actually became arginine, but arginine was clearly being made. These findings indicate that ornithine is made by the *sup-3* mutant from glutamate in the presence of arginine even though acetylglutamate synthase is presumably still feedback sensitive. The continued synthesis of arginine shows again that CPS-A is not directly inhibited by arginine. In relation to the first point, continued ornithine synthesis in the mitochondrion in *sup-3* strains might impair mitochondrial transport of arginine by transinhibition and therefore might protect acetylglutamate synthase from feedback inhibition. This is highly speculative, but it would at least explain the continued ability of *sup-3* cells to form ornithine. A critical test of this hypothesis will be difficult.

Removal of Arginine

A major observation of Goodman and Weiss (unpublished experiments) concerned the speed with which arginine synthesis resumed in wild type when arginine was removed from the medium. It was observed some time ago that arginine synthesis resumed quickly after short-term exposure to arginine (96). This would be expected of a feedback-inhibitable system in which repression did not have time to occur. However, after long-term growth in arginine, removal of arginine was followed by a very slow resumption of glutamate entry into the pathway (96). This was not expected because none of the enzymes prior to ornithine is repressible. (In these experiments, an *ota* mutation was used so that glutamate entering the pathway could be confined to, and measured as, arginine intermediates.)

It was then asked whether the slow resumption of arginine synthesis after long-term growth in arginine reflected repression of enzymes after all or the maintenance of a feedback-inhibited state. The *sup-3* strain (which displays only partial feedback inhibition of ornithine synthesis) was again used. This strain, unlike the wild type, quickly resumed flux of glutamate into the pathway after both short- and long-term growth in arginine. The observation suggested strongly that wild type was slow in resuming ornithine and arginine synthesis not because of prior repression, but because feedback inhibition was maintained. Thus, in the wild type, and possibly in the *sup-3* mutant, mitochondrial arginine concentrations may reach high levels after prolonged growth in the presence of this amino acid, and it might take some time to deplete this pool when cells are returned to minimal medium.

In *N. crassa*, transfer of an arginine-grown mycelium to minimal medium leads to reduced arginine catabolism (239), although in some cases it continues for some time (Goodman and Weiss, unpublished experiments). This is correlated with the immediate return of the rate of ornithine transaminase formation to normal (237) as noted in a previous section. This suggests that arginine, the inducer, was largely confined to the vacuole. The experiment was also done with an arginine auxotroph (239). While catabolism ceased, growth of the auxotroph continued at a normal rate until the vacuolar pool was exhausted. This showed that the vacuole regulated arginine concentration in the cytosol so that protein synthesis proceeded normally, but catabolism did not occur. The narrow range of arginine concentration in the cytosol is therefore maintained despite huge variations in the vacuolar content of arginine. The data as a whole suggest strongly that the vacuole is the proximate regulator of cytosolic arginine concentrations. Derepression comes into play only when the vacuole is depleted of arginine, and feedback inhibition is exerted only when the vacuole is unable to remove arginine from the cytosol quickly.

CONCLUSION

The differences in the ways *N. crassa* and *S. cerevisiae* accomplish similar metabolic steps and transitions emphasize the differences likely to be encountered among other organisms. Owing to its simple lifestyle and extraordinary genetics, *S. cerevisiae* has been used to study and to exemplify the molecular biology of eucaryotes at a cellular level. It is appealing because its regulation has many features in common with *E. coli* and can be dealt with by using a similar vocabulary. However, the temptation to generalize from this "honorary bacterium" to other eucaryotes, even other fungi, carries risks that must be tested by study of other organisms. Thus, while *S. cerevisiae* will continue to reveal the molecular basis of individual mechanisms, it is unlikely that knowledge of complex functional systems can be safely generalized.

If we consider *N. crassa*, it is clear that an enormous amount of metabolic change can be accomplished independently of differential gene expression. Instead, the structure of the cells and the design of enzymes (significant, it must be said, in *S. cerevisiae* as well) are sufficient to allow *N. crassa* to adapt rapidly and effectively to changing circumstances. A single mycelium may meet many environments as it grows in nature. Mechanisms that equip vegetative cells to deal locally and transiently with a variety of conditions in the absence of significant growth or gene expression must surely contribute to fitness.

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